



AGRICULTURAL RESEARCH INSTITUTE
PUSA

PROCEEDINGS
OF THE
ROYAL SOCIETY OF LONDON
SERIES B
CONTAINING PAPERS OF A BIOLOGICAL CHARACTER
VOL. XCIX.

LONDON:
PRINTED FOR THE ROYAL SOCIETY AND SOLD BY
HARRISON AND SONS, LTD., ST. MARTIN'S LANE,
PRINTERS IN ORDINARY TO HIS MAJESTY

MAY, 1926.

LONDON:

**HARRISON AND SONS, LTD., PRINTERS IN ORDINARY TO HIS MAJESTY
ST. MARTIN'S LANE.**

CONTENTS.

SERIES B. VOL. XCIX.

PAGE

No. B 694.—December 1, 1925.

On the Supposed Pluri-segmental Innervation of Muscle Fibres. By L. N. Katz. Communicated by Prof. A. V. Hill, F.R.S.	1
Lactic Acid in Mammalian Cardiac Muscle.—Part I. The Stimulation Maximum. By L. N. Katz and C. N. H. Long. Communicated by Prof. A. V. Hill, F.R.S.	8
Lactic Acid in Mammalian Cardiac Muscle.—Part II. The Rigor Mortis Maximum and the Normal Glycogen Content. By H. J. G. Hines, L. N. Katz, and C. N. H. Long. Communicated by Prof. A. V. Hill, F.R.S.	20
Lactic Acid in Mammalian Cardiac Muscle.—Part III. Changes in Hydrogen-ion Concentration. (Preliminary Note.) By L. N. Katz, Phyllis Tookey Kerridge, and C. N. H. Long. Communicated by Prof. A. V. Hill, F.R.S. ...	26
The Effect of Light on the Circulation. By D. T. Harris. Communicated by Prof. E. H. Starling, F.R.S.	28
The Mechanism of Muscular Contraction. By W. E. Garner, Reader in Physical Chemistry at University College, London. Communicated by Prof. A. V. Hill, F.R.S.	40
The Effects of Calcium and Potassium Ions on Urine Secretion, as Studied in the whole Animal. By L. Brull and F. Eichholtz. Communicated by Prof. E. H. Starling, Foulerton Professor of the Royal Society.	57
The Secretion of Inorganic Phosphate by the Kidney.—II. Influence of the Pituitary Gland and of the Wall of the Third Ventricle. By L. Brull and F. Eichholtz. Communicated by E. H. Starling, F.R.S., Foulerton Professor of the Royal Society	70
Hydrolysis of Phosphoric Esters by the Kidney <i>in Vivo</i> . By F. Eichholtz (Rock- efeller Travelling Fellow), Robert Robison and L. Brull. Communicated by E. H. Starling, F.R.S., Foulerton Professor of the Royal Society	91

No. B 695.—January 1, 1926.

Address of the President, Sir Charles Sherrington, O.M., at the Anniversary Meeting, November 30, 1925	107
A Critical Statistical Study of Experimental Data on the Effect of Minute Electric Currents on the Growth Rate of the Coleoptile of Barley. By F. G. Gregory and L. Batten. Communicated by Prof. V. H. Blackman, F.R.S.	122

Measurements of the Reversing Spiral in Cotton Hairs. By W. Lawrence Balls, F.R.S., and H. A. Hancock.....	130
A Spirometer Method of Studying Continuously the Gaseous Metabolism of Man during and after Exercise. By K. Furusawa. Communicated by Prof. A. V. Hill, F.R.S.	148
Muscular Exercise, Lactic Acid, and the Supply and Utilisation of Oxygen.—Part XIII. The Gaseous Exchanges of Restricted Muscular Exercise in Man. By K. Furusawa. Communicated by Prof. A. V. Hill, F.R.S.	155
Muscular Exercise, Lactic Acid, and the Supply and Utilisation of Oxygen.—Part XIV. The Relation in Man between the Oxygen Intake during Exercise and the Lactic Acid Content of the Muscles. By C. N. H. Long. Communicated by Prof. A. V. Hill, F.R.S.	167
The Hydrogen-Ion Concentration and Oxidation-Reduction Potential of the Cell-Interior before and after Fertilisation and Cleavage: A Micro-Injection Study on Marine Eggs. By Joseph Needham, M.A., Ph.D., Fellow of Caius College, Cambridge, and Dorothy Moyle Needham, Girton College, Cambridge, Beit Memorial Research Fellow. Communicated by Sir Frederick Hopkins, F.R.S.	173

No. B 696.—February 1, 1926.

Chlorocruorin: A Pigment allied to Hæmoglobin. By H. Munro Fox, Fellow of Gonville and Caius College, Cambridge, and Balfour Student in the University. Communicated by Prof. J. Stanley Gardiner, F.R.S. (Plate 1).....	199
Effects of Ultra-Violet Radiation upon Involuntary Muscle, and the Supposed Physiological Interference of Visible Rays. By Y. Azuma and Leonard Hill, F.R.S.	221
The Early Development of Cavia: Note on Associated Remains of Previous Placentation. By Norman H. W. MacLaren. Communicated by Prof. T. H. Bryce, F.R.S. (Plate 2)	230
Studies on the Relation of Gonadic Structure to Plumage Characterisation in the Domestic Fowl.—I. Henny-Feathering in an Ovariectomised Hen with Active Testis Grafts. By A. W. Greenwood and F. A. E. Crew, Animal Breeding Research Department, University of Edinburgh. Communicated by Prof. R. C. Punnett, F.R.S. (Plates 3 and 4)	232
The Induction of Melanism in the Lepidoptera and its Subsequent Inheritance. By J. W. Heslop Harrison, D.Sc., and F. C. Garrett, D.Sc. Communicated by Prof. E. W. MacBride, F.R.S.	241

No. B 697.—March 1, 1926.

The Diffraction Method of Measuring the Diameters of Erythrocytes. By W. G. Millar, M.B., Ch.B., F.R.M.S. Communicated by Sir E. Sharpey Schafer, F.R.S.	264
---	-----

The Metabolism of the Diabetic Individual during and after Muscular Exercise. By K. S. Hetzel and C. N. H. Long. Communicated by Prof. A. V. Hill, F.R.S.	279
A Closed-Circuit Heart-Lung Preparation.—Effect of Alterations in the Peri- pheral Resistance and in the Capacity of the Circulation. By I. de Burgh Daly. Communicated by Prof. E. H. Starling, F.R.S.	306
The Heat Liberated by the Beating Heart. By E. Fischer (Rockefeller Fellow). Communicated by Prof. A. V. Hill, F.R.S.	326

No. B 698.—April 6, 1926.

Experimental Studies on the Differentiation of Embryonic Tissues growing <i>in vivo</i> and <i>in vitro</i> .—I. The Development of the Undifferentiated Limb-Bud (<i>a</i>) when Subcutaneously Grafted into the Post-Embryonic Chick and (<i>b</i>) when Cultivated <i>in vitro</i> . By T. S. P. Strangeways, Lecturer in Special Pathology, University of Cambridge, and Honor B. Fell, Ph.D., Beit Memorial Research Fellow. Communicated by Sir William Hardy, F.R.S. (Plates 5–13)	340
The Meiotic Phase in Certain Mammals. By C. E. Walker, D.Sc., Department of Cytology, University of Liverpool. Communicated by J. B. Farmer, F.R.S. (Plates 14–16)	366
The Effect of Insulin on the Dextrose Consumption of Perfused Skeletal Muscle. By Charles H. Best. Communicated by Dr. H. H. Dale, Sec. R.S.	375
Further Micro-Injection Studies on the Oxidation-Reduction Potential of the Cell- Interior. By Joseph Needham, M.A., Ph.D., Fellow of Caius College, Cambridge, and Dorothy Moyle Needham, Girton College, Cambridge, Beit Memorial Research Fellow. Communicated by Prof. Sir Frederick Hopkins, F.R.S.	383
The Mechanism of Ciliary Movement.—V. The Effect of Ions on the Duration of Beat. By J. Gray, King's College, Cambridge. Communicated by Prof. Stanley Gardiner, F.R.S.	398

No. B 699.—May 1, 1926.

The Influence of Muscle Work on Metabolism in Varying Conditions of Diet. By E. P. Cathcart, F.R.S., and W. A. Burnett (late Lawrence Student), Institute of Physiology, Glasgow University	405
The Life-Cycle of the Nodule Organism, <i>Bacillus Radicicola</i> (BERR.), in Soil and its Relation to the Infection of the Host Plant. By H. G. Thornton, B.A., and N. Gangulee, Ph.D. Communicated by Sir John Russell, F.R.S.	427
Ultra-Violet Radiation and Metabolism, with a New Method for Estimating Metabolism. By J. Argyll Campbell. Communicated by Dr. Leonard Hill, F.R.S. (Plate 17)	451
A Study of Certain Forms of Inhibition and Acceleration of Hæmolysis. By Eric Ponder. Communicated by Sir E. Sharpey Schafer, F.R.S.	461

The Isolation of Some Hitherto Undescribed Products of Hydrolysis of Proteins. —Part II. By S. B. Schryver, Professor of Biochemistry, and H. W. Buston, Research Assistant, Imperial College of Science and Technology. Com- municated by Sir John B. Farmer, F.R.S.	476
The Secretion of Pituitrin in Mammals, as shown by Perfusion of the Isolated Kidney of the Dog. By E. B. Verney, Beit Memorial Research Fellow. Communicated by Prof. T. R. Elliott, F.R.S.	487

OBITUARY NOTICES.

Sir Archibald Geikie (with portrait)	i
Thomas George Bonney (with portrait)	xvii
Sir William Maddock Bayliss (with portrait)	xxvii
Arthur Dendy (with portrait)	xxxiii
Frank Evers Beddard	xxxvi
James Sykes Gamble (with portrait)	xxxviii
Sidney Harris Cox Martin	xliv
James Fairlie Gemmill	xl v
Index	xlix

PROCEEDINGS OF THE ROYAL SOCIETY.

SECTION B.—BIOLOGICAL SCIENCES.

On the Supposed Pluri-segmental Innervation of Muscle Fibres.

By L. N. KATZ.*

(Communicated by Prof. A. V. Hill, F.R.S.—Received July 23, 1925.)

(From the Department of Physiology, University College, London.)

Cattell and Stiles (1) have recently claimed to show that the majority of skeletal muscle fibres, at least in frogs, have a pluri-segmental innervation. The existence of a similar innervation in mammalian muscle fibres has also been asserted by Agduhr (2).

The evidence supporting this theory falls into two categories. The first is based on the histological findings after Wallerian degeneration has occurred in the axons rising from one spinal segment. In such cases muscle fibres were found in which one axon and motor end-plate showed signs of degeneration, and the other, when two were present, did not. Agduhr, who made the observation, concluded that the degenerated axon came from the segment whose motor roots he had cut 56 to 144 hours previously, and the undegenerated axon from another spinal segment. He was careful, however, to point out in his last paper that the total number of such doubly (or trebly) innervated fibres could not be adduced from his experiments.

These observations are liable to the criticism that the time allowed for the degeneration was not long enough to allow one to assume with certainty that degeneration had occurred in all the axons coming from the severed motor root. Even, however, if we admit the validity of Agduhr's observation, we cannot be certain whether this pluri-segmental innervation obtains in most of the fibres or is merely a chance variation. Kulchitsky (3) has recently

* Fellow in Medicine of the National Research Council, U.S.A.

expressed the opinion that every muscle fibre, at least in the frog, is supplied with only one terminal apparatus (motor), and that this, regardless of how complex it may be, comes from one nerve fibre.

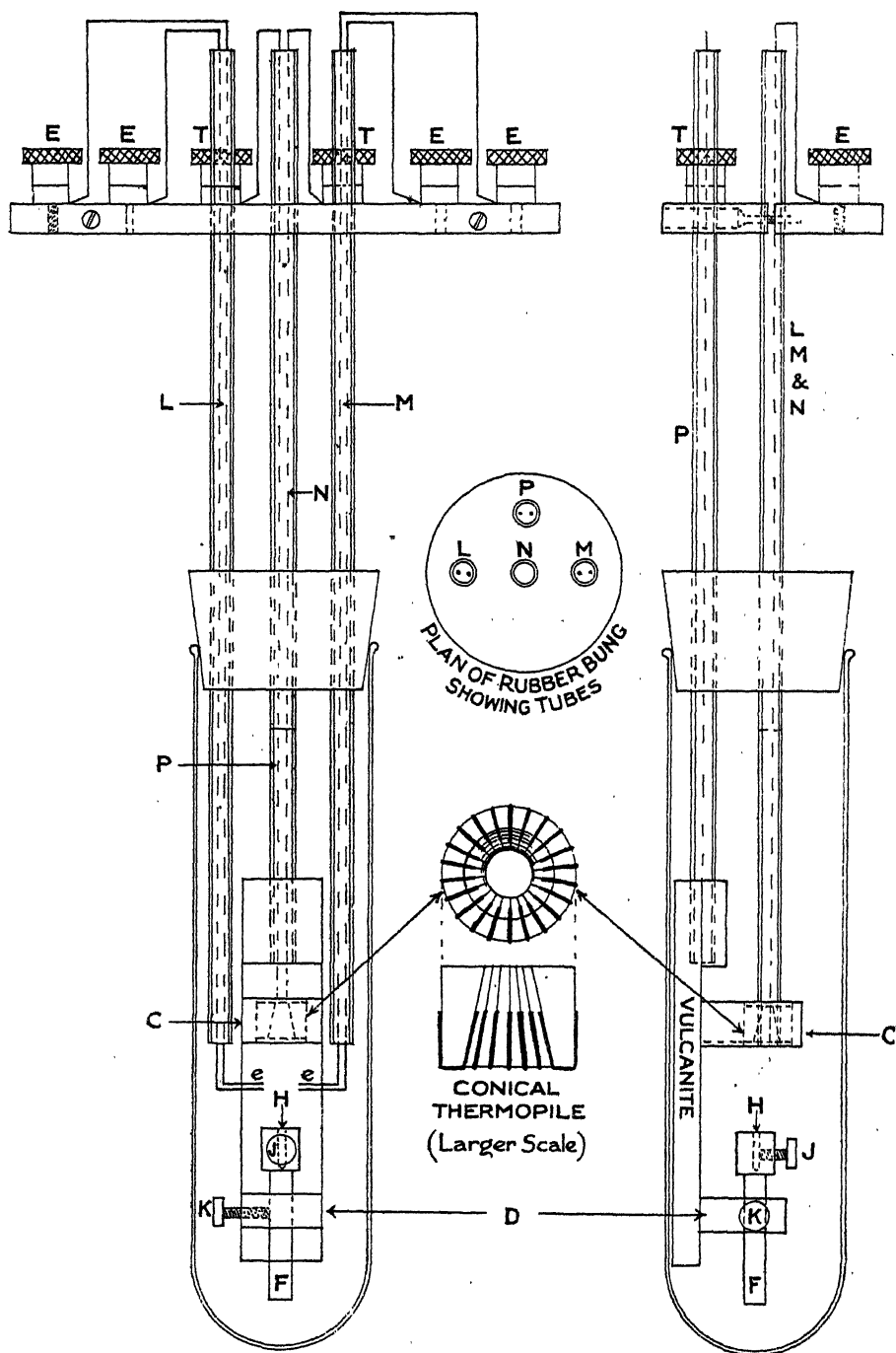
The second line of evidence is based on the work done and the tension developed by a muscle (*a*) when one of the motor roots from which it is innervated is stimulated, and (*b*) when the complete nerve is stimulated. Douglas and Gotch (4) showed that the stimulus which gave a maximal response of the gastrocnemius when applied to the sciatic nerve of a muscle-nerve preparation only produced a sub-maximal response when applied to one of the roots from which the sciatic nerve is formed. Agduhr (2) found the same thing in the fore-limb muscles of the cat: he noted, however, that the force of the contraction obtained by stimulating the various motor roots separately exceeded the contraction given by stimulating them all at the same time. Cattell and Stiles (1) repeated the experiment on frog's muscle-nerve preparations, and found that the sum of the tensions developed in an isometric tetanus, on stimulating each of the nerve roots separately, exceeded the tension developed when the entire sciatic nerve was excited. The ratio had an average value of 1.7 to 1. They concluded that 50 per cent. of the nerve fibres innervate 85 per cent. of the contractile units, in other words about 70 per cent. of the muscle fibres, have a double segmental innervation.

This latter line of evidence, however, is not above criticism, since work and tension are not necessarily additive quantities in the sense of being directly proportional to the number of muscle fibres excited. In a muscle such as the gastrocnemius, the fibres are not parallel but run in a complex series of twisted curves, each for a distance considerably less than the full length of the muscle. The tension developed in a contraction is the resultant and not the sum of the individual tensions of the elementary fibres. When only a small number of the fibres are active, especially if lying close together in the muscle, they will tend to exert a force in a direction lying more or less along their mean position. They will bulge the rest of the muscle out so that the contracting element will tend to lie along the line joining the upper and lower tendons. When, however, the whole muscle contracts, such a change of position will not be able to occur, the various fibres pulling in a variety of directions will exert a resultant force by no means equal to the sum of the forces of the several fibres. The mean direction of the contracting fibres of any particular section of the muscle may be able to turn itself parallel to the long axis of the muscle as a whole when one nerve root alone is stimulated, whereas when both sections of the muscle are stimulated together, each may to some extent be pulling against the other,

and the resultant force in the direction of the muscle as a whole may be less. It is possible, therefore, to suppose that the result described by Cattell and Stiles may be due not to any pluri-segmental innervation, but to the minute structure of the muscle.

It seemed desirable, therefore, to test the theory of pluri-segmental innervation by a method which measures a quantity directly proportional to the number of muscle fibres participating in the contraction. A determination of the heat offers a ready method of doing this. The objections which may be raised against the use of tension, or height of contraction, as criteria of the number of fibres contracting do not apply to this method, since heat has no directional quality and depends, when the contraction is isometric and the initial length constant, only on the number of fibres participating actively in the contraction.

The experiments of Cattell and Stiles were repeated in this investigation, but with the difference that heat was measured as well as tension. A special thermopile was constructed for the purpose, mounted in a chamber on the lines of the one described by Fenn (5). The essential parts of the apparatus are shown in the figure. The thermopile proper, which is conical in shape, is in the upper vulcanite shelf C. It is 1 cm. long, with an opening at the bottom of 5 mm. diameter and tapering towards the top, where the opening is 2 mm. in diameter. These dimensions were found by trial to give the best fit to the distal portion of the belly of the gastrocnemius of medium-sized frogs. The thermopile contains 24 constantan-silver couples wound round a thin vulcanite cone, its resistance being 12 ohms. A vulcanite rod F is fitted in the lower shelf D; in the top of this rod is a hole H to receive the femur of the muscle-nerve preparation. The screws J and K secure respectively the femur in the holder and the latter in the shelf in the position desired. The holder does not lie along the axis of the thermopile, but a few millimetres to one side, since it was found by trial that the best fit of the gastrocnemius in the thermopile is obtained in this way. Four glass tubes, L, M, N and P, pass through the rubber bung. The central one, N, which is in line with the thermopile, allows the thread attached to the tendon of the gastrocnemius to pass out to the tension lever. The two tubes, L and M, contain the wires leading from the two pairs of platinum electrodes, *e, e*, on which the two roots of the sciatic nerve are laid to the terminals, E, E, E, E. These electrodes are adjustable; as a rule they are placed $1\frac{1}{2}$ to 2 cms. apart. The fourth tube P contains the wire leads of the thermopile running to the terminals T, T, and also carries the vulcanite block containing the thermopile. The entire apparatus is fitted into a large

Diagram of Thermopile ($\frac{1}{2}$ actual size).

tube or bottle, which serves as a "moist chamber," and submerged beneath well-stirred water in a large Dewar flask.

The tension developed was recorded on smoked paper by an isometric tension lever of fairly short period. In the earlier experiments a Paschen galvanometer was employed; in the latter a modified Broca, as described by Downing(6). The galvanometers were critically damped, the deflection times when employing the thermopile being about 4 to 6 seconds, and the sensitivity about 5×10^{-10} ampères per millimetre on the scale. In many of the experiments, additional resistance had to be used in order to reduce the size of the deflection.

After the usual interval from the time of setting up the apparatus the two component parts of the sciatic nerve were stimulated with a tetanising current of short duration ($1/20$ th to $\frac{1}{2}$ second) from a Porter coil. The duration of the stimulus was regulated either by a Keith-Lucas rotating contact-breaker (for the longer tetani), or by a Keith-Lucas spring pendulum contact-breaker (for the short tetani). The current was directed to either of the two nerve components or to both in series by means of a mercury commutator to which the platinum electrodes were connected. The strength of the current was so regulated that a maximal response was obtained when both roots of the sciatic nerve were stimulated simultaneously. The isometric lever was so adjusted as to give a small initial tension in the muscle.

A series of readings was then taken, the usual order of stimulation being (1) both components together, (2) component A, (3) component B, (4) both components together, &c. The results were not converted into absolute units, but left as scale divisions for heat and fiftieths of an inch for tension. The readings obtained with each preparation were tabulated and averaged; the averages were then converted into percentages, the heat and tension developed when both components were stimulated together being the standard 100 per cent. A long series of observations was made in each experiment.

The results of 16 experiments on 16 different preparations are given in Table I.

This table shows that whereas the sum of the tensions obtained by stimulating the two components separately is considerably more than the tension obtained by stimulating both together, thus confirming Cattell and Stiles, the sum of the heats is almost precisely equal to the heat obtained by stimulating both components together. On the average ($T_A + T_B$) exceeds the tension obtained by stimulating both together by 41 per cent., which excess is not as great as that given by Cattell and Stiles, and there is a great variability from one preparation to another, the figures ranging from 17 per cent. to 77 per

Table I.

H_A = average heat on stimulating component A.
 H_B = average heat on stimulating component B.
100 = average heat on stimulating A and B together.
 T_A = average tension on stimulating component A.
 T_B = " " " " " B.
100 = " " " " " A and B together.

Exp. No.	H_A	H_B	$H_A + H_B$	T_A	T_B	$T_A + T_B$
1	27	72	99	51	86	137
2	47	64	111	82	95	177
3	35	53	88	54	63	117
4	34	62	96	67	90	157
5	6	86	92	—	—	—
6	12	97	109	22	100	122
7	11	85	96	—	—	—
8	13	84	97	—	—	—
9	33	80	113	—	—	—
10	29	82	111	42	81	123
11	30	71	101	60	80	140
12	24	73	97	54	85	139
13	44	62	106	76	80	156
14	17	81	98	70	89	159
15	33	71	104	45	85	130
16	17	81	98	57	74	131
Average	26	75	101	57	84	141

cent. If these figures were an index of the number of fibres doubly innervated they would mean that anything from 17 to 77 per cent. of the muscle fibres have a pluri-segmental innervation. The results, however, which were obtained in the same way by a measurement of the heat, preclude such an interpretation, for here ($H + H_B$) is only 1 per cent. greater, on the average of 16 preparations, than the heat obtained by stimulating both components together.

We may conclude, therefore, that the excess of tension found in the case of separate stimulation is due to some characteristic which does not affect the heat, and it is natural to attribute this to the vector quality of the mechanical response. We may further conclude that there is no evidence of a double segmental innervation of the muscle fibres of the gastrocnemius of the frog.*

Summary.

The finding of Cattell and Stiles is confirmed that in the frog, when the two components of the sciatic nerve are stimulated in succession in an isometric

* Beritoff's (7) results, if true, must have another explanation than the one he gives in view of these findings.

tetanus, the sum of the tension developed is considerably greater than the tension produced when both components are stimulated simultaneously.

Their claim that this indicates that the majority of the muscle fibres in the frog's gastrocnemius are innervated from more than one spinal segment is criticised. An alternative explanation is suggested, based on the fact that the resultant of two forces is not necessarily their sum.

A crucial test was applied to the theory of pluri-segmental innervation by repeating Cattell and Stiles's experiments, employing the heat production as a criterion of the number of muscle fibres activated. Heat has no directional quality, and the deflections obtained should be simply additive, provided that the contraction is isometric and the initial tension constant.

A special thermopile was constructed for the purpose.

The total heat developed on stimulating the two components of the sciatic nerve separately is the same, within experimental error, as the heat produced when the two components are stimulated simultaneously. This indicates that there is no pluri-segmental innervation of frog's muscle fibres apart from possible infrequent and chance variations.

It is a pleasure to acknowledge my indebtedness to Prof. A. V. Hill for his suggestion of this research, and for his interest in the work, to Mr. A. C. Downing for constructing the thermopile used and for supplying me with the drawing of it which is given in the text, and to Mr. Downing and Mr. J. L. Parkinson for their assistance.

During the progress of this research we learned that Mr. J. F. Fulton, at Oxford, was also investigating the same subject. It is interesting that he has reached independently, and on different grounds, the same conclusion as arrived at here (8).

After this paper was written an article by De Boer appeared on the same subject in the 'Journal of Physiology' (vol. 60, p. 214, 1925). Although his conclusions agree with ours, his evidence is not entirely above criticism; for it might be argued that the veratrine acts not on the muscle fibre itself but on the nerve endings, in which case his findings would only prove that fatigue of some nerve endings does not affect the character of the response of the rest, which Cattell and Stiles have already shown. His results, therefore, do not eliminate the possibility that the muscle fibres have a pluri-segmental innervation.

REFERENCES.

- (1) Cattell and Stiles, 'Amer. Journ. Physiol.,' vol. 69, p. 645 (1924).
 - (2) Agduhr, 'Anat. Anzeiger,' vol. 49, p. 1 (1916); vol. 52, p. 273 (1919).
 - (3) Kulchitsky, 'Journ. Anat.,' vol. 59, p. 1 (1924).
 - (4) Gotch, 'Journ. Physiol.,' vol. 28, p. 412 (1902).
 - (5) Fenn, 'Journ. Physiol.,' vol. 58, p. 175 (1924).
 - (6) Downing, 'Journ. Scient. Instruments,' vol. 2, p. 255 (1925).
 - (7) Beritoff, 'Pflüger's Arch.,' vol. 205, p. 455 (1924).
 - (8) Fulton, 'Roy. Soc. Proc.,' B, vol. 98, p. 493 (1925).
-

Lactic Acid in Mammalian Cardiac Muscle.—Part I. The Stimulation Maximum.

By L. N. KATZ* and C. N. H. LONG.†

(Communicated by Prof. A. V. Hill, F.R.S.—Received July 25, 1925.)

(From the Department of Physiology, University College, London.)

Introduction.—The earlier work of Fletcher and Hopkins (1), and the more recent researches of A. V. Hill (2), Meyerhof (3) and their collaborators on the rôle of lactic acid in muscular contraction, have shown that oxygen is not required for the production of lactic acid, but only for its removal during the recovery process. This fact, coupled with the ability of a skeletal muscle to respond to stimulation until its lactic acid content is many times the resting value, enables the skeletal muscle to draw on its future supply of oxygen and to go into "oxygen debt." The question naturally arises, are these facts also true of the heart muscle? Can the heart respond to stimulation when it has accumulated as large a concentration of lactic acid as is found in the skeletal muscle? In other words, How does the stimulation maximum of lactic acid of the heart compare with that of the skeletal muscle?

A priori certain differences might be expected in view of the differences in the activity and nutrition of cardiac and skeletal muscles. In the first place the heart is not called upon to increase its energy output to anything like the extent of the skeletal muscle; nor does the heart have any long periods of comparative rest. It is always active and is contracting roughly one-third of the time. In addition the heart is provided with a very efficient circulation and has the first call on the oxygenated blood. For these reasons there seems to be no apparent need for the heart to have the ability of accumulating lactic acid to the same extent as the skeletal muscle; indeed, in view of the necessity for its constant activity, it would seem dangerous for it not to possess a mechanism by which the removal of lactic acid keeps pace with its production.

With this in mind we have made (1) a comparison of the effects of oxygen lack for a short interval on these two tissues, and (2) a determination of the stimulation maximum of lactic acid in both types of muscle.

* Fellow in Medicine, National Research Council, U.S.A.

† Working on behalf of the Industrial Fatigue Board, Medical Research Council.

I.—THE EFFECT OF OXYGEN LACK ON THE CONTRACTION OF MAMMALIAN
CARDIAC AND SKELETAL MUSCLE.

Method.—Observations were made both on isolated mammalian preparations and on decapitated animals: 28 experiments were performed on 10 animals (two rats, six rabbits, and two cats).

Rats and rabbits were used for the isolated preparations. The animal was killed in the usual way by a blow on the back of the neck, and the auricle or ventricle to be used was quickly removed and placed in oxygenated Ringer-Locke solution kept in a bath at 37° C. This was done by one of us while the other removed as quickly as possible one or more of the thin muscles from the forelimb and placed it in the same Ringer-Locke solution. The two tissues were then placed in two separate cylinders containing oxygenated Ringer-Locke solution and kept at 37° C. in a water bath. The arrangement was such that, when necessary, oxygen or nitrogen could be bubbled through the solutions. The chambers could also be rapidly and simultaneously emptied and refilled with fresh Ringer-Locke solution. This was done when the gas was changed, so that there should be no delay in the response due to residual gas dissolved in the solution. The muscles were attached to light isotonic levers and were stimulated with break shocks through platinum electrodes at rates varying from 40 to 100 per minute.

It was found that even with the greatest care the skeletal muscle preparation would not keep in good condition for more than a short time: consequently, the observations were repeated on decapitated cats and rabbits. Sherrington's method of decapitation was used (we are indebted to Dr. N. B. Dreyer for demonstrating the technique). Artificial respiration was instituted, the chest opened and the heart freed from its pericardium. The records of the ventricular contraction were made by attaching the apex to an ordinary lever. Cotton wool was loosely packed beneath the heart to prevent the shifting of its base with respiration and the consequent distortion of the records. The gastrocnemius muscle of one of the limbs was exposed and freed from its surroundings, care being taken not to damage the blood supply. The tendon was cut and fastened to a lever; the knee and ankle joints were clamped to render the limb immobile. The heart and muscle were then stimulated as in the isolated preparations. The electrodes on the heart were placed on the auricles in the region of the sinus node, and so arranged that they moved with the heart and so offered no impediment to its contraction. In some experiments the heart was not stimulated but allowed to beat naturally. One or more electric

bulbs was placed about 6 in. from the muscle and heart to keep them warm. Care was taken that the muscles did not become dry.

The experiment itself in the case of the isolated preparations consisted in shutting off the oxygen supply, quickly changing the Ringer-Locke solution in the two chambers, and then bubbling nitrogen through the fresh solutions. In some experiments the oxygen supply was merely cut off, the Ringer-Locke solutions not being changed. In the decapitated animals the same effect was produced by shutting off the artificial respiration.

Results.—The records obtained in this way, although not demonstrating any new principles, do show the differences in the behaviour of heart and skeletal muscle when exposed to conditions of oxygen want. The following are a few typical examples.

Fig. 1 was obtained on isolated preparations of a rabbit's heart and skeletal

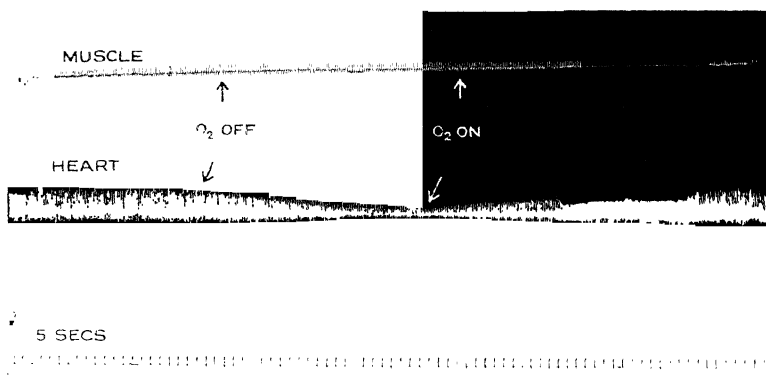


FIG. 1 (reduced $\frac{2}{3}$).—Myograms of isolated preparations of rabbit's auricle and skeletal muscle stimulated 110 times a minute, showing the effect of oxygen lack for a short period of time.

muscle. The heart was beating spontaneously, but on commencing the stimulation it responded to each stimulus. On turning off the oxygen the amplitude of the heart beat quickly decreased, but recovered equally quickly when the oxygen was turned on again. It will be noted that the skeletal muscle was unaffected by these changes and continued to respond quite as well in the absence of oxygen as it did in its presence.

Fig. 2 is from an experiment on a decapitated cat. Here both muscle and heart were stimulated 80 times a minute with break shocks. The results obtained here are identical with those in fig. 1, *i.e.*, rapid dilatation and failure when respirations ceased, and equally rapid recovery when the artificial respira-

tion was started again. The muscle as in the previous experiment was unaffected by the cutting off of the artificial respiration.

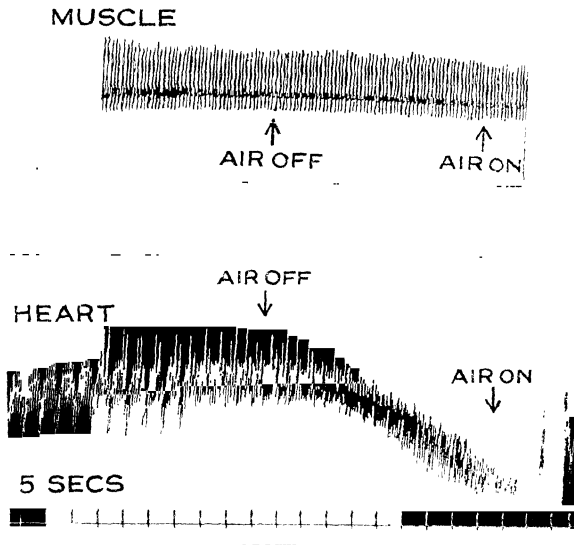


FIG. 2 (reduced $\frac{1}{2}$).—Myograms of heart and skeletal muscle (decapitated cat) stimulated 80 times a minute, showing effect of oxygen lack for short period of time.

Fig. 3 is also from a decapitated cat. In this experiment the muscle was stimulated 100 times a minute, while the heart was allowed to beat with its

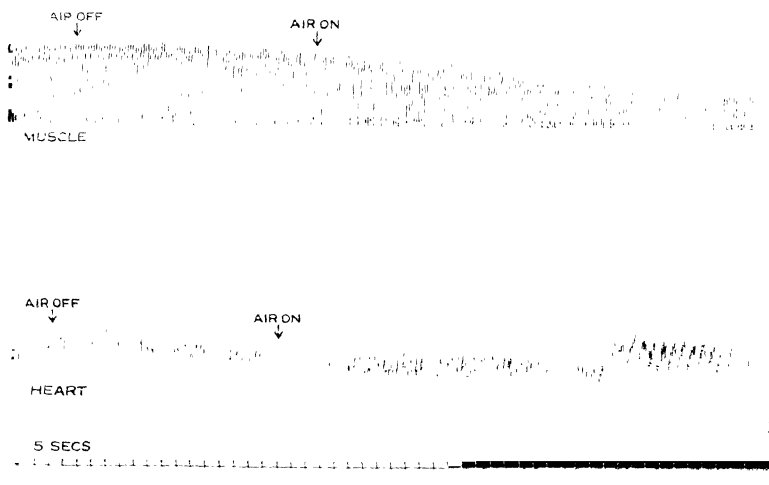


FIG. 3.—Same as fig. 2, except that heart was beating naturally.

own rhythm. The skeletal muscle showed a typical fatigue curve with an initial "treppe." The slope of the fatigue curve was unaffected by the cutting off of the oxygen supply. The heart on the other hand first beat irregularly and extra systoles appeared, then it slowed and stopped, and in this case took a long time to recover, probably because the asphyxia had been carried too far.

The differences were equally striking to the eye. The heart, whether stimulated or beating naturally, was always affected in a short time by the cutting off of its oxygen supply. Its contractions became feebler, it dilated, and conduction was so slowed that in many cases a "peristaltic wave" could be seen to pass over the ventricles instead of the normal synergic contraction. In the naturally beating heart the rate decreased extra systoles were present and block of varying degrees appeared. The heart recovered almost as soon as the oxygen supply was turned on again, unless the asphyxia was continued until diastolic standstill or ventricular fibrillation occurred. In contrast, the skeletal muscle showed hardly any changes while these were occurring in the heart.

These observations are not new, but so far as we know no previous attempt has been made to compare the effect of oxygen lack on these two tissues in the same animal and under comparable conditions. When such a direct comparison is made the difference is very obvious. There can be no doubt that the heart is more dependent on its contemporary supply of oxygen than skeletal muscle, which, for such short intervals of time, is quite independent of its presence or absence.

II.—THE LACTIC ACID STIMULATION MAXIMUM OF MAMMALIAN HEART AND SKELETAL MUSCLE.

If the heart be dependent on its contemporary supply of oxygen to such an extent as the above observations show, then it cannot go into debt for large amounts of oxygen and cannot accumulate much lactic acid before ceasing to function properly. This being so, the stimulation maximum of lactic acid in the heart should be much less than in the skeletal muscle, in which values ranging from 0.2 to 0.3 per cent. (1, 4) are readily obtained. We have, therefore, made a comparison of the lactic acid stimulation maximum in the heart and skeletal muscle.

Method.—The animals (cats and rabbits) were anaesthetised, in the early experiments with ether, in the later ones with ether and chloralose. They were then decapitated, either by Sherrington's method or by one which we devised. In the latter method the chest was opened, artificial respiration

instituted and the vessels to the head quickly clamped and tied off; first the arteries coming from the arch of the aorta, and then the superior vena cava and azygos vein. In this way the circulation to the brain was interrupted, and after a gasp or two the animal was physiologically decapitated. When ether alone was used a bottle of it was placed in the circuit of the artificial respiration apparatus until the operation was completed. There seems to be less shock in the latter method than in Sherrington's.

After the decapitation one gastrocnemius muscle was removed as a control. In some experiments the control muscle was removed before decapitation. After removal of the muscle the femoral artery of that limb was clamped. The second gastrocnemius was prepared and stimulated as before described. The tendon, however, was not cut, no record was obtained, and the contractions were made as isometric as possible by clamping the limb and paw in a suitable position. In most of the experiments the muscle was stimulated with break shocks at rates from 70–200 per minute. In two experiments a short tetanus was used instead. The platinum electrodes were in most cases fastened directly into the muscle, and when the latter showed signs of fatigue they were moved into a different position. This was repeated, and the secondary of the induction coil was gradually advanced as the muscle became tired, until no further response was obtained. In three experiments the muscle was stimulated indirectly through the sciatic nerve. When completely fatigued the muscle was removed and its lactic acid content determined. In most experiments the circulation to the muscle was intact until the latter was removed, but in a few cases the femoral artery was clamped immediately before removal.

The next step in the experiment was to fatigue the heart. In order to do this the descending aorta, or in many cases the ascending aorta, was tied off, so that the heart for practical purposes was pumping blood only through the pulmonary circuit and its own arteries. The artificial respiration was then stopped, and when the heart showed signs of fatigue it was stimulated through the platinum electrodes until it no longer responded. In four experiments only were we able to exhaust the heart without asphyxia, in two instances anoxemia was induced with cyanide (1/1,000), and in two others adrenaline (1/10,000) was injected to speed up the heart rate. When the ventricles had ceased to contract the heart was removed and the lactic acid content of the ventricular muscle determined.

The Estimation of Lactic Acid in the Muscles.—It is not an easy matter to obtain accurate and reliable estimations of the lactic-acid content of muscles, particularly of mammalian muscles. As soon as a muscle is deprived of its oxygen supply, or is injured in any way, lactic acid is rapidly formed. Fletcher

and Hopkins (1) were the first to point out, in the case of amphibian muscle, the errors to which a neglect of this fact may lead. Alcohol is an admirable killing agent, but its use for this purpose is one of the best ways of causing the *post mortem* production of lactic acid if the temperature is not at 0° C. In all our estimations, therefore, 95 per cent. alcohol was used as the killing agent, but it was placed in porcelain basins, which were immersed in a freezing mixture of NaCl, or crystalline CaCl₂, and crushed ice.

This precaution is not sufficient, for when a mass of muscle weighing several grammes is immersed in alcohol, even at this temperature, although the surface layers of the tissue are killed at once, the resulting coagulation of the proteins forms a layer through which the alcohol only penetrates slowly. The interior of the mass is consequently not killed immediately, and continues to produce lactic acid for a considerable length of time. This error was avoided by chopping the muscle up finely with a pair of blunt-nosed scissors as soon as it was immersed in the alcohol. A large number of surfaces are exposed to the action of the alcohol by this procedure, while the low temperature (0° to — 5° C.) prevents the injury from producing large amounts of lactic acid. It is important, however, to make the interval between the removal of the muscle from the animal and its immersion as short as possible. Usually one of us quickly cut out the heart or gastrocnemius, then removed the tendon (or, in the case of the heart, any adherent fat, the auricles, valves, and vessels) with a sharp pair of scissors, and wiped off as much of the blood as possible. The tissue was next placed on the balance and rapidly weighed to the nearest centigramme, then dropped into the alcohol and quickly chopped up by the other. The whole procedure occupied about 1 to 2½ minutes.

Even with these precautions, there is a certain formation of lactic acid due to injury to the tissue; we believe, however, that our technique gives slightly lower values for this quantity than does that of other observers; the mean value for the skeletal muscle being 0.06 per cent., and for the cardiac muscle 0.03 per cent. (*cf.*, Table I). The reason for this difference in the resting value for the two tissues we do not know. It may be explained by the hypothesis (a) that the heart *in vivo* contains less lactic acid than the skeletal muscle, or (b) that the skeletal muscle may contain substances not present in the heart which are estimated as lactic acid by this method.

For the rest, the method used consisted of a combination of those of Meyerhof (4) and Clausen (5). After standing in the alcohol in the freezing mixture for half an hour, the supernatant alcohol was poured off through a fluted filter paper, and the muscle ground up with coarse sand. The mass of

disintegrated muscle and sand was placed on the filter paper, and the washings from the mortar and other dishes poured on to it. The complete filtrate was refiltered through the mass three times, to ensure complete extraction of the lactic acid, and the residue washed once with fresh alcohol. The alcoholic extract was then slowly evaporated to dryness on a not too vigorously boiling water bath. The residue (fat, etc.) was then extracted, while still on the water bath, with about 15 c.c. of a saturated solution of ammonium sulphate. The ammonium sulphate solution was added in two portions, the first being filtered off through a Büchner funnel, and the extraction repeated with the second portion.

The filtrate was washed into a 50 c.c. separating funnel, 4 c.c. of 4 per cent. NaOH added, and extracted three times with 6 c.c. of pure crystallised benzene. The solution was then neutralised and heated on the water bath to drive off the traces of benzene and ammonia. It was then ready for oxidation. The solution was made up to a definite volume, usually 50 c.c., and an aliquot part of it taken and oxidised.

We took an aliquot part for oxidation because the estimation of smaller quantities of lactic acid (up to 10 mgms.) has already been studied by one of us (6), and the loss due to the procedure determined. It seemed more advisable to subdivide the solution so as to bring the amount of lactic acid oxidised down to these limits, rather than to oxidise much larger quantities (such as were present in the entire extract) since the loss in doing this may be quite different from that sustained with smaller quantities. Some additional notes as to the technique may be found in the paper referred to above (6).

We have made control experiments in which known amounts of lactic acid were added to the muscle, and have found that the recovery of lactic acid from the whole process of extraction and estimation is 75 per cent. This agrees with the figures given by Meyerhof (4), so we have used it in calculating our lactic acid results.

Results.—The content of lactic acid found in the normally beating heart and in the resting skeletal muscle are given in the following table (Table I).

The quantities of lactic acid found in the resting muscle and in the normally beating heart are formed, no doubt, chiefly by the manipulations necessary in the method. They compare well in the case of the skeletal muscle with the figures obtained by other observers (Meyerhof (4), Fletcher and Hopkins (1)).

Table I.—Lactic Acid Content of Normally Beating Heart and Resting Skeletal Muscle.

Cat, No.....	2.	3.	6.	8.	9.	12.	13.	14.	25.	27.	Rabbit.	Mean.
Lactic acid : skeletal muscle, gram. per cent.	0.040	0.041	0.075	0.083	0.053	0.035	0.065	0.074	—	—	0.040	0.056
Lactic acid : heart, gram. per cent.	—	—	—	—	0.031	—	—	—	0.034	0.028	0.022	0.028

The stimulation maxima under our experimental conditions are given in the next table (Table II). Except where otherwise stated the heart was exhausted by asphyxia and stimulation.

Table II.—Stimulation Maximum of Lactic Acid in Heart and Skeletal Muscle.

Animal.	Lactic acid in grammes per cent.		Notes.
	Skeletal Muscle.	Heart.	
Cat A	0.118	0.045	Skeletal muscle, electrodes not moved during stimulation. " " " " " " " " " " " " " " " "
" 1	0.099	0.043	
" 2	0.157	0.089	
" 3	0.171	0.047	
" 17	0.213	0.130	
Rabbit 2	0.224	0.099	Skeletal muscle stimulated through sciatic nerve. Adrenaline added to heart.
" 4	0.164	0.085	
Cat 6	0.277	0.077	Skeletal muscle stimulated through sciatic nerve. "
" 8	0.314	0.112	
" 10	0.276	0.085	
" 14	0.270	0.074	
" 20	0.189	0.075	
" 31	0.228	0.042	
" 32	0.302	0.059	
" 33	0.316	0.100	
" 21	—	0.052	
" 22	—	0.050	
" 23	—	0.067	Heart exhausted by cyanide and stimulation. " " " " " " " "
" 28	—	0.048	
" 30	0.272	0.051	
Mean....	0.252	0.072	Heart driven to exhaustion with air on. Skeletal muscle stimulated directly.

The stimulation maximum of lactic acid in the skeletal muscle varied from 0.189 to 0.314 gram. per cent., when stimulated directly. When a muscle

was stimulated through the sciatic nerve the content of lactic acid was less (0.164 to 0.224 grm. per cent.), probably owing to the greater susceptibility of the nerve endings. We have also found that if the electrodes are not moved about on the muscle, then the local accumulation of lactic acid appears to set up a block which interferes with the spread of the excitation to the other parts of the tissue, the average concentration consequently being lower (in four experiments the values ranged from 0.099 to 0.171 grm. per cent.). This local block has already been pointed out by other observers (Stiles and Catell (7)).

The stimulation maximum in the heart is much smaller than in the skeletal muscle as Table II will show. There is a large variation for different animals but never do we find the heart accumulating amounts of lactic acid comparable to the skeletal muscle, although a number of different factors, such as adrenaline, cyanide, exhausting in the presence of adequate artificial respiration, were tried. The ratio of the absolute concentrations in skeletal muscle and in ventricular muscle is about 0.252 to 0.072 or $3\frac{1}{2}$ to 1. If we compare only the *increase* in lactic acid content (above the resting or normally active muscle) we find that in the heart the lactic acid content has increased $2\frac{1}{2}$ times, while in the skeletal muscle it has increased $4\frac{1}{2}$ times. The ratio of the *increase* in lactic acid content, in skeletal and cardiac muscle, is also about $4\frac{1}{2}$ to 1.

Inasmuch as 7 grm. of lactic acid require 1 litre of oxygen for its removal (*cf.* (8)), the average oxygen debt in our series would be 0.28 c.c. of O_2 per gramme in the case of the skeletal muscle, and only 0.063 c.c. of O_2 per gramme in cardiac muscle.

Discussion.

Several possibilities have been suggested to explain our result that the heart ceases to beat when its lactic acid concentration is much less than that at which the skeletal muscle fails. (a) The difference might be due to the fact that the actual muscle substance in the heart is, so to speak, more diluted with inactive connective tissue, than in the skeletal muscle. This point has already been investigated by Schenck (9), who finds that while the connective tissue forms roughly 10 per cent. of the heart, it forms only 2 per cent. of a skeletal muscle. This difference, however, is not large enough to be of any significance in explaining our results.

(b) The difference might be due to the fact that a comparatively local disturbance in the ventricle, such as might accompany a local accumulation of lactic acid, will set up ventricular fibrillation, or (if it be in the region of the common bundle) a heart block: whereas such a local accumulation in the case of the skeletal muscle will not interfere with the activity of the rest, under

the conditions of stimulation in most of our experiments. The total quantity of lactic acid found would then be small because the greater part of the heart muscle would not be in an entirely exhausted condition. Although this factor does operate we do not believe that it alone will suffice, because direct electrical stimulation of various points on the ventricles was always tried in order to be certain that all the muscle fibres were exhausted. Furthermore, no noticeable difference in the lactic acid content was found between the hearts which failed (i) by diastolic standstill and (ii) by ventricular fibrillation.

(c) The low stimulation maximum in the heart might be due to the fact that the heart does not have sufficient lactic acid precursor in the form of glycogen, etc., to enable it to go on contracting for very long in the absence of oxygen. A *résumé* of the literature gave contradictory results on this point. Some investigators have found more glycogen in the heart than in the skeletal muscle, others less. We have reinvestigated the matter, using normal healthy cats under the same conditions as in our lactic acid experiments, and have found that the normally beating heart has less glycogen than the resting skeletal muscle. The results are given in detail in Part II. We have ascertained, however, that the heart can have as much as 0.35 grm., per cent. lactic acid in "rigor mortis" (*cf.* Part II). It cannot, therefore lack precursor to produce the stimulation maximum of lactic acid, which in our series never exceeded 0.14 grm. per cent. ..

(d) The difference in the stimulation maximum would be explained if the heart were more susceptible to hydrogen-ions than the skeletal muscle. Under such conditions the critical pH might be reached in the heart, even by the addition of the comparatively small quantities of lactic acid found. The recent work of Andrus and Carter (10), Andrus and Drury (11) and Andrus (12) have shown that the isolated heart is very susceptible to changes in pH , and that when the reaction becomes too acid not only is the force of the beat lessened, but the rate of conduction of the impulse is slowed and the excitability of the heart diminished. It seemed essential, therefore, to determine the critical pH in these two tissues. This we have done with the co-operation of Mrs. Kerridge, M.Sc., of this department, using the recently-described glass electrode (13) which she has perfected. The full details of the method and the results will be published later, but a preliminary note is given in Part III below. At present we will merely point out that we have found the critical pH to be less acid in the heart than in the skeletal muscle.

Smaller quantities also of lactic acid would suffice to reach the critical pH in the heart, if its buffering power were poor as compared with skeletal muscle.

We have investigated this also and found such to be the case. This also is referred to in Part III.

We have concluded then that the low stimulation maximum of lactic acid in the heart is due primarily to the greater susceptibility of this organ to a rise in hydrogen-ion concentration, together with the fact that its poor buffering power allows the critical value of pH to be reached with but a comparatively small addition of free acid.

Summary.

(1) Methods are described for comparing the effects of short intervals of oxygen lack on mammalian heart and mammalian skeletal muscle. Evidence is given to show that the heart is dependent on its contemporary oxygen supply, and that it fails rapidly in its absence; the skeletal muscle, on the other hand, is independent at first of its contemporary oxygen supply being unaffected by short intervals of oxygen lack. It is concluded that the heart cannot go into "oxygen debt" to anything like the extent of the skeletal muscle.

(2) A method is described for determining the stimulation maximum of lactic acid in heart and in skeletal muscle. The data obtained in 20 cats and rabbits show that the stimulation maximum in the heart is much less than in skeletal muscle. This also points to the fact that the heart cannot go into "oxygen debt" to the same extent as skeletal muscle. The mean value of the lactic acid stimulation maximum for a skeletal muscle was 0.252 gm. per cent., and for the heart less than one-third of this, viz., 0.072 gm. per cent. The maximum "oxygen debts" of these two tissues should, therefore, be in the same ratio.

(3) An explanation for the difference in the lactic acid stimulation maximum was sought for and several possibilities discussed. It was concluded, chiefly as a result of the evidence given in a Preliminary Note in Part III, that the comparatively low level of the lactic acid stimulation maximum is due primarily to the greater susceptibility of the heart to an accumulation of hydrogenions, and to the poorer buffering power of the heart. Such small quantities of lactic acid as we have found in the heart are sufficient to bring the fibres to the critical pH , at which further activity is impossible, because (1) this critical pH is less acid than in the skeletal muscle, and, therefore, less change of pH is necessary; and (2) the heart muscle is less well buffered, so that a given change of pH is caused by less acid production.

We wish to express our gratitude to Prof. A. V. Hill, F.R.S., for his suggestions and criticisms of this investigation.

BIBLIOGRAPHY.

- (1) Fletcher and Hopkins, 'Jl. of Physiol.,' vol. 35, p. 247 (1907).
- (2) A. V. Hill *et al.*, 'Jl. of Physiol.' and 'Proc. Roy. Soc.' (numerous papers, 1914-25).
- (3) Meyerhof *et al.*, 'Arch. f. d. gesam. Physiol.,' and 'Biochem. Zeitsch.' (numerous papers, 1920-25).
- (4) Meyerhof, 'Arch. f. d. gesam. Physiol.,' vol. 182, pp. 232-920 (1920).
- (5) Clausen, 'Jl. Biol. Chem.,' vol. 52, p. 263 (1922).
- (6) Long, 'Proc. Roy. Soc.,' B, vol. 96, p. 449 (1924).
- (7) Catell and Stiles, 'Am. Jl. of Physiol.,' vol. 69, p. 651 (1924).
- (8) Hill, Long and Lupton, 'Proc. Roy. Soc.,' B, vol. 97, p. 96 (1924).
- (9) Schenck, 'Arch. f. d. gesam. Physiol.,' vol. 202, p. 322 (1924).
- (10) Andrus and Carter, 'Heart,' vol. 11, p. 97 (1924).
- (11) Drury and Andrus, 'Heart,' vol. 11, p. 389 (1924).
- (12) Andrus, 'Jl. of Physiol.,' vol. 59, p. 361 (1924).
- (13) Kerridge, 'Bioch. Journal' (*in press*, 1925).

Lactic Acid in Mammalian Cardiac Muscle.—Part II. The Rigor Mortis Maximum and the Normal Glycogen Content.

By H. J. G. HINES, L. N. KATZ* and C. N. H. LONG.†

(Communicated by Prof. A. V. Hill, F.R.S.—Received July 25, 1925.)

(From the Department of Physiology, University College, London.)

I. The Rigor Mortis Maximum.

In Part I of this paper two of us have shown that the stimulation maximum is different in mammalian cardiac and skeletal muscle. The heart muscle has only about one-third of the lactic acid concentration of the skeletal muscle, when both no longer respond to stimulation. We suggested as one possible explanation the fact that the heart normally might not contain as much lactic acid precursor as the skeletal muscle. In the present investigation the total amount of lactic acid produced in ordinary rigor mortis,‡ and in the rigor mortis induced by the use of buffered phosphate solutions of caffeine, was determined. Cats were used as in the preceding experiments.

* Fellow in Medicine, National Research Council, U.S.A.

† Working for the Industrial Fatigue Board, Medical Research Council.

‡ The term *rigor mortis*, we admit, ill expresses the condition of the heart after death as there is no sign comparable to that of the skeletal muscle.

In the first series of observations the ordinary rigor mortis formation of lactic acid was determined. The usual procedure was to take a limb (or a pair of limbs) from a cat used in the stimulation maximum experiment, and to place it for three hours in an incubator kept at 37° C. Along with the muscle were put either both ventricles or the portion that remained after a sample had been taken for determination of the stimulation maximum. After incubation the tissues were treated as described in Part I, and their lactic acid content measured.

In the other series of experiments the lactic acid maximum, after treatment with caffeine solution, was determined by taking portions of heart and skeletal muscle and chopping them up finely in known volumes of buffered phosphate solution of pH.8 to which caffeine had been added to make a 0.2 per cent. solution. It is very probable from the results obtained by Hill and Hartree (5) and Meyerhof (6) that such treatment will break down all the lactic acid precursor in a muscle into lactic acid. The muscles were incubated in the solutions for three hours at 37° C. The lactic acid in the solutions and that remaining in the muscles were then determined separately, the solution being removed by filtration, and its volume measured. Sufficient sodium tungstate and sulphuric acid were added to remove any protein that might be present in the solution, and the latter again filtered. To the known volume of filtrate, copper sulphate and a suspension of calcium hydroxide were added to remove glucose. After standing $\frac{1}{2}$ hour a further filtration was done and an aliquot part of the filtrate taken to determine the lactic acid content. From this the total amount of lactic acid in the solution could be calculated. The procedure for determining the lactic acid in the muscle itself was the same as described in Part I. It is interesting to note that under these conditions the greater portion (80 per cent.) of the lactic acid diffused into the caffeine solution.

The results of both series of experiments are given in Table I.

Table I.—Rigor Mortis.

I. Muscles incubated 3 hours at 37° C.

Lactic acid in grams per 100 grm. muscle.								
Cat No.	9.	12.	13.	14.	15.	16.	18.	Mean.
Heart	0.24	0.31	0.18	0.20	0.26	0.20	0.22	0.23
Skeletal muscle	0.56	0.51	0.38	0.48	0.53	0.66	0.51	0.52

Ratio—Skeletal muscle : Heart, 2.3 : 1.

Table I—*continued*.

II. Muscles incubated 3 hours at 37° C. with 0.2 per cent. caffeine and -phosphate solution (pH . 8).

Lactic acid (grams per 100 grm. muscle.)						
Cat No.	19.	20.	23.	26.	31.	Mean.
Heart	0.32	0.34	0.26	0.36	0.31	0.32
Skeletal muscle.....	0.50	0.61	0.55	0.74	0.61	0.60

Ratio—Skeletal muscle : Heart, 1.9 : 1.

It will be seen from this table that the rigor mortis maximum of lactic acid in the heart is about $\frac{1}{2}$ that in the skeletal muscle. This ratio agrees very well with that obtained by Miss Arning at Manchester (unpublished) in the case of chloroform rigor in frogs' and tortoises' hearts and skeletal muscles.

Discussion.—When we examine the figures for the greatest possible amount of lactic acid that can be found in the two tissues, and compare them with the maximum figures for the *stimulation maximum* of lactic acid, we find that the difference in lactic acid precursor cannot be the cause of the difference in the stimulation maxima. (See Table II.)

Table II.

	Stimulation Maximum of Lactic Acid (grams per 100 grams of muscle).	Buffered Caffeine Rigor Maximum of Lactic Acid (grams per 100 grams of muscle).	Percentage of Maximum reached at the Stimu- lation Maximum.
Heart	0.07	0.32	Per Cent. 22
Skeletal muscle	0.25	0.60	42

The stimulation maximum in the heart (under our experimental conditions) is only 22 per cent. of the total possible production, whereas the skeletal muscle under similar conditions has utilised 42 per cent. of the lactic acid precursor present. The fact that in the heart the stimulation maximum of lactic acid is so much further removed from the absolute maximum induced by caffeine than in the skeletal muscle is explained by the results of Part III, namely, that the poorer buffering power of the cardiac muscle as compared with the

skeletal muscle results in the critical pH being reached with the production of less lactic acid in the heart than in the skeletal muscle. This effect is further enhanced by the fact that the critical pH is less acid in the heart than in the skeletal muscle.

II. *Normal Glycogen Content.*

If in the heart the precursor of lactic acid be glycogen, as it is known to be in the case of the skeletal muscle (Meyerhof(4)), then we should expect to find the ratio of glycogen content in the two tissues the same as that of the buffered caffeine rigor maxima of lactic acid. A study, however, of the literature on the subject of the comparative glycogen contents of the two tissues reveals a large number of conflicting observations. Recently Schenck (2) has determined the glycogen contents of the cardiac and skeletal muscles of dogs, and found that on the average the ratio of glycogen in the heart to that in skeletal muscle is of the order of 2 : 3. Macleod and Prendergast (1) also have shown that in the well-fed, healthy animal (dogs and rabbits) it is customary to find more glycogen in the skeletal muscle than in the heart. Some of the other investigators are in agreement with Schenck and with Macleod and Prendergast (1), but others maintain the reverse, namely, that the heart normally contains more glycogen than skeletal muscle. No doubt this conflict of opinion is due to two causes. Firstly, sufficient care has not always been taken in the past to prevent breakdown of glycogen in the muscles during the preliminary stages of its extraction. It is just as important to prevent glycogenolysis as it is to prevent an undue formation of lactic acid in similar determinations of the latter. Secondly, the condition of the animals on which the determinations have been made has not always been all that could be desired. Macleod and Prendergast (1) have shown clearly that starvation depletes the glycogen reserves of the skeletal muscles while leaving the state of the heart practically unaltered. In starved animals there may be, in consequence, an exact reversal of the ratio found in the two tissues of well-fed animals. In the present investigation, therefore, the glycogen content of the two tissues was determined in as normal and well-fed animals as could be procured. Care also was taken to diminish glycogenolysis to a minimum.

In this set of determinations the cat was anæsthetised with ether. The gastrocnemius on one side was exposed, dissected out and removed, the femoral artery on that side then being clamped. The chest was next opened and the heart quickly excised, the auricles, vessels and fat removed with sharp scissors and the blood squeezed out. Both tissues immediately after excision were weighed and then dropped into porcelain dishes containing alcohol kept at

— 1 to — 5° C. by placing the dishes in a freezing mixture. The muscles were immediately chopped up into fine bits with scissors. Up to this stage the technique is essentially that used in our control lactic acid determinations (Part I). The whole procedure from the time of excision to the mincing of the muscles took 1 to 2 minutes. The heart in most cases was still beating when put into the alcohol.

After standing ice-cold for half an hour the alcohol was poured into hot 60 per cent. KOH and allowed to boil off. The tissue was then placed in the hot KOH and left to dissolve for two to three hours. The solution was allowed to cool and the glycogen was precipitated with alcohol according to Pflüger's method, the solution being allowed to stand over night in order to get complete precipitation. It was next filtered through Gooch crucibles and the precipitate washed with 60 per cent. alcohol. The contents of the Gooch crucible was then transferred, asbestos and all, to tubes containing 20 c.c. of 2·5 per cent. HCl. The tubes were placed on a boiling water bath and the solution allowed to hydrolyse for four to five hours. The solution was again filtered through the same Gooch crucible in order to remove the asbestos and then washed with distilled water. The filtrate was neutralised and made up to standard volume. The reducing sugar was then determined by Bertrand's method and the figure multiplied by 0·907 to give the glycogen content. This is calculated as $(C_6H_{10}O_5)_n$. In all cases the heart and muscle of a given animal were treated in an identical manner.

The results are given in Table III.

Table III.—The Glycogen content of normal heart and muscle.

Glycogen content (grams per 100 grm. muscle).											
Cat. No.	51.	52.	53.	54.	55.	56.	57.	58.	59.	60.	Mean.
Heart	0·140	0·197	0·041	0·057	0·235	0·160	0·169	0·174	0·070	0·140	0·138
Skeletal muscle	0·455	0·306	0·460	0·627	0·411	0·469	0·535	0·781	0·770	0·725	0·554

Ratio—Skeletal muscle : Heart, 4 : 1.

It will be seen at once from this table that the heart of the normal well-fed cat has only 1/4 the glycogen content of the skeletal muscle.

Discussion.

The ratio of glycogen content in heart and skeletal muscle is different from the ratio of the rigor maximum content of lactic acid in the two tissues. There is less glycogen in the heart than available lactic acid as the following table (Table IV) shows.

Table IV.—Comparison of available lactic acid and normal glycogen content of heart and skeletal muscle.

	"Buffered" Caffeine Rigor Maximum of Lactic Acid in grams, per cent.	Glycogen Content in grams, per cent.	Difference.
Heart	0.32	0.14	0.18
Skeletal muscle	0.60	0.55	0.05
Ratio.....	1.9 to 1	4 to 1	

The difference between the two figures is 0.05 in the case of the skeletal muscle and 0.18 in the case of the heart. The differences give us an idea, if the figures be correct, of the relative amount of lactic acid precursor in the form of hexose phosphate or some related compound, since the figures given in the first column of Table I are a measure of all the lactic acid precursor plus the free lactic acid present in the resting stage, while those in the second column give an idea of how much lactic acid can come from glycogen. The greater difference between the two figures in the heart may be interpreted to mean that the heart has more lactic acid precursor in the form of hexose phosphate or some other similar compound than has the skeletal muscle. In this connection we note that Schenck (3) has made determinations in dogs of the "lactacidogen" in the two tissues and finds more in the heart than the muscle, although the difference is not as large as is suggested by our results.

The validity of this contention could be further tested by comparing the glycogen content of the heart, when it no longer responds to stimulation, and in ordinary and caffeine rigor, with the lactic acid content under the same conditions, to see whether or not the disappearance of glycogen keeps pace with the formation of lactic acid as in skeletal muscle (Meyerhof (4)). This point merits further investigation.

Summary.

1. The rigor mortis maximum, or the caffeine rigor maximum, of lactic acid in the heart is only half that in the skeletal muscle.

2. A lack of lactic acid precursor is not the cause of the relatively low stimulation maximum of lactic acid in the heart.

3. The glycogen content of the heart is much smaller than that of skeletal muscle in normal well-fed animals (cats).

4. There is in the heart a greater discrepancy between the resting glycogen content and the lactic acid produced in rigor mortis, than there is in skeletal muscle. This indicates that a greater portion of the lactic acid precursor of the heart is in some other form than glycogen. It is possible that this may be a hexose phosphate or some related compound.

We wish to express our gratitude to Prof. A. V. Hill, F.R.S., for his helpful advice and criticism.

BIBLIOGRAPHY.

- (1) Macleod and Prendergast, 'Roy. Soc. Proc.,' Canada, vol. 15, Section V, p. 37 (1921).
- (2) Schenck, 'Arch. f. d. gesam. Physiol.,' vol. 202, p. 315 (1924).
- (3) Schenck, 'Arch. f. d. gesam. Physiol.,' vol. 202, p. 329 (1924).
- (4) Meyerhof, 'Arch. f. d. gesam. Physiol.,' vol. 85, p. 11 (1920).
- (5) Hartree and Hill, 'Journ. of Physiol.,' vol. 58, p. 441 (1924).
- (6) Meyerhof, 'Arch. f. d. gesam. Physiol.,' vol. 188, p. 143 (1921).

Lactic Acid in Mammalian Cardiac Muscle.—Part III. Changes in Hydrogen-ion Concentration. (Preliminary Note.)

By L. N. KATZ,* PHYLLIS TOOKEY KERRIDGE,† and C. N. H. LONG.‡

(Communicated by Prof. A. V. Hill, F.R.S.—Received July 25, 1925.)

(From the Department of Physiology, University College, London.)

Experiments have been made to determine (1) the hydrogen-ion concentrations of cardiac and skeletal muscle minced in the cold (*a*) under normal conditions, (*b*) after stimulation to fatigue, and (*c*) in rigor mortis, and (2) the change of hydrogen-ion concentration following the addition of known amounts of lactic acid. The measurements were made by means of the glass electrode method (Kerridge (1)).

The results given are quantitatively only preliminary in character, pending

* Fellow in Medicine, National Research Council, U.S.A.

† Working on behalf of the Medical Research Council.

‡ Working on behalf of Industrial Fatigue Board, Medical Research Council.

(i) the repetition of the experiments on a larger number of animals, and (ii) the more accurate estimation of corrections due to dilution with saline, &c. The experiments so far made clearly indicate that :—

(i) The pH 's of cardiac and of skeletal muscles stimulated to fatigue are different. The difference is of the order of $0.2\ pH$, the skeletal muscle being the more acid.

(ii) The pH 's of cardiac and of skeletal muscles in rigor mortis are different ; the difference being of the order of $0.4\ pH$, the skeletal muscle again being the more acid.

(iii) Cardiac and skeletal muscle have different buffering powers. The ratio of the concentration of lactic acid added, to the change in pH , varies with the pH , rising to a maximum at a pH of approximately 6.3 , when the ratio of the buffering power of skeletal to that of cardiac muscle is about $2 : 1$.

Calculations of the amounts of lactic acid developed in the muscles, made from the pH determination and the buffer curves, give a value for the ratio of the increase of concentration of lactic acid developed in skeletal muscle on stimulation to that in cardiac muscle as $3 : 1$, as compared with $4\frac{1}{2} : 1$ as found directly in Part I. A similar calculation for rigor mortis gives a ratio of $2.2 : 1$, as compared with $2.3 : 1$ found in Part II. The agreement between the two methods in the latter case indicates that there is no acid other than lactic acid formed in the muscles.

This work is being continued, and an account of it will be published later in detail.

REFERENCES.

- (1) Kerridge, 'Biochem. Journ.' (*in press*) (1925).
-

The Effect of Light on the Circulation.

By D. T. HARRIS.*

(Communicated by Prof. E. H. Starling, F.R.S.—Received July 25, 1925.)

[From the Physiology Institute, University College, London.]

Introduction.

It has long been the practice in clinical work to apply measures for the lowering of blood pressure in hyperpiesis. The immediate purpose of this investigation is to examine the basis upon which phototherapy stands as a suitable agent for the lowering of blood pressure.

The original application of light baths by Finsen is an expression of his belief in this form of therapy. Among the earliest quantitative observations are those of K. A. Hasselbalch and Jacobäus (1), who found that repeated exposures to the total radiations of the carbon arc caused a fall in the blood pressure of about 10 per cent. in the medium-sized arteries. Bach (2) obtained similar results in 105 out of the 109 cases exposed to the quartz mercury-vapour lamp. These observers state that the lowering of blood pressure persisted for about a month. More recent observations by H. Königsfeld (3) corroborate these earlier findings, with the exception of a few individual variations.

It is not always clear in the earlier records whether the results apply equally to normal and hyperpietic individuals. Bach (2) discriminated between these and definitely stated that he found no effect of the radiations in people with normal blood pressure; this is also the experience of Sequeira and O'Donovan (4) and other workers. In the mixed patients examined by Rollier (5) and his colleagues a fall of blood pressure was the usual result of solar radiations.

So far as the writer is aware, the above measurements of blood pressure appear to have been made by sphygmomanometric methods, employing palpation and perhaps more recently auscultation. The amount of manipulation involved in these methods is in itself sufficient in the human subject to cause variations in the blood pressure far exceeding those resulting from artificial and solar radiations. For this reason it became necessary to investigate the effect of the various forms of radiations upon the blood pressure

* Working for the Biological Action of Light Committee of the Medical Research Council.

by graphic methods, the utmost precaution being taken to eliminate all disturbing factors. Further, plethysmographic tracings were arranged in an endeavour to determine the mode of action of radiations on the circulation.

Method.

Normal men and women, volunteers from among the medical students, were the first subjects to be examined. Any members exhibiting hyper-irritability, or lack of normal endurance, were immediately rejected since standard conditions were difficult of attainment in such individuals. The subject, with back laid bare, was placed in a padded arm-chair, slightly tilted back so that there might be no tendency for the body to slip down, the feet being placed on an inclined plane firmly fixed so that the knees were semi-flexed. The weight of the head was taken by an ophthalmic chin-rest. The pneumatic cuff of a sphygmomanometer was fitted on the left arm, the forearm being wrapped in cotton-wool. This cuff communicated with the inflating pump, which could be shut off, and with the lower chamber of the pressure-equalising cylinder of an oscillograph (fig. 1), designed by Dr. F. L. Soler, of Buenos Aires, and lent to me by Prof. Starling.

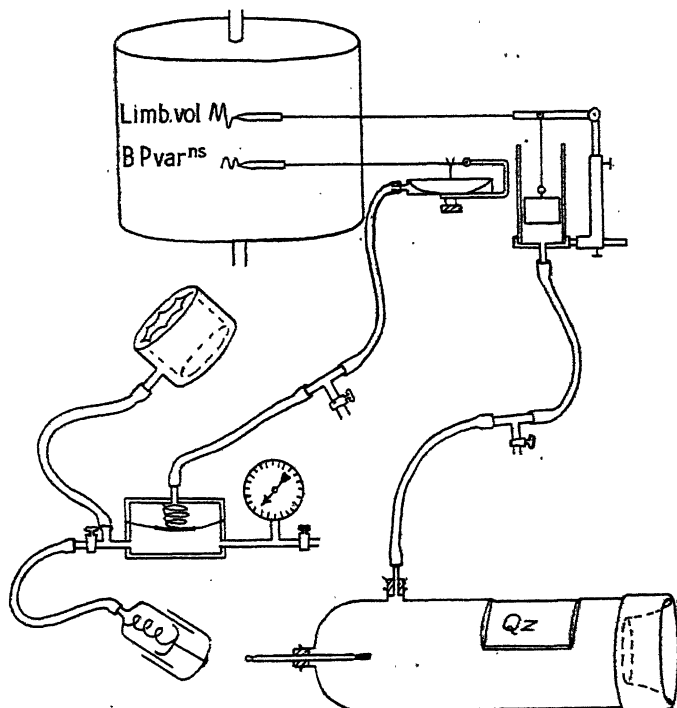


FIG. 1.—Sphygmomanometric oscillograph and Plethysmograph with quartz window.

The upper chamber was connected to a small recording tambour, the tension of whose membrane could be finely adjusted, and this was usually arranged so that a deflection of the writing-point through 1 cm. corresponded to a 5 mm. head of mercury in the armlet. It was found that with this degree of sensitivity large variations were recorded with simple psychological tests.

Experiments were performed with the purpose of determining whether it was justifiable to apply this apparatus to the recording of variations in blood pressure, especially in view of the fact that the cuff was inflated to a pressure considerably below the systolic pressure. The cuff was adjusted to the femoral region of a dog, connected as in fig. 1 and inflated to a pressure of 105 mm. mercury; definite pulsations were discernible before applying the writing point to the drum. The animal's blood pressure recorded 165 mm. in the carotid artery, and this was artificially raised by a small intravenous injection of adrenalin. The correspondence of the curves obtained by these two different methods of recording blood pressure is shown in fig. 2, and was regularly experienced both in pressor and depressor responses.

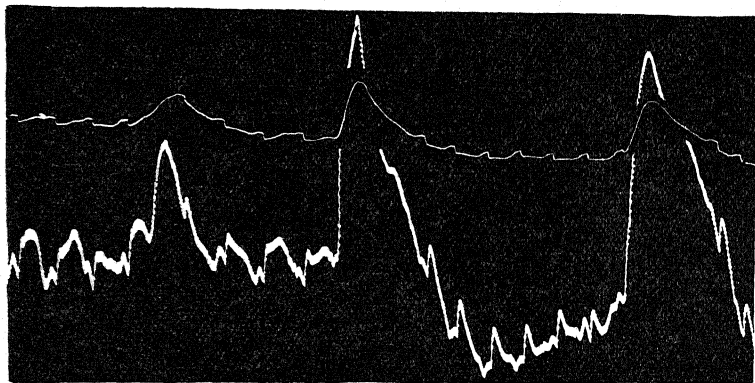


FIG. 2.—Effect of adrenalin in a dog on the sphygmomanometric oscillogram (upper curve) and on the kymogram.

The sensitivity of the apparatus is, obviously, much less when used in a dog because of the difficulty of suitably applying a human armlet to an animal's limb. If, however, it is desired to employ this method of recording variations of blood pressure in the intact animal, this may be done by means of the metallic cuff devised by Kolls (6) for dogs combined with his compound lever recorder.

To demonstrate the sensitivity of the apparatus in the case of man, simultaneous measurements were periodically made by the usual auscultatory method with a mercury sphygmomanometer. For speed and precision in

determining the latter, the rubber-protected chest-piece of the stethoscope was firmly strapped to the arm immediately below the cuff on the brachial artery. The time-signal was operated by an assistant, who recorded on paper the systolic blood pressure; these values were then plotted on squared paper and transferred to the tracing in the manner shown in fig. 3. The lag due

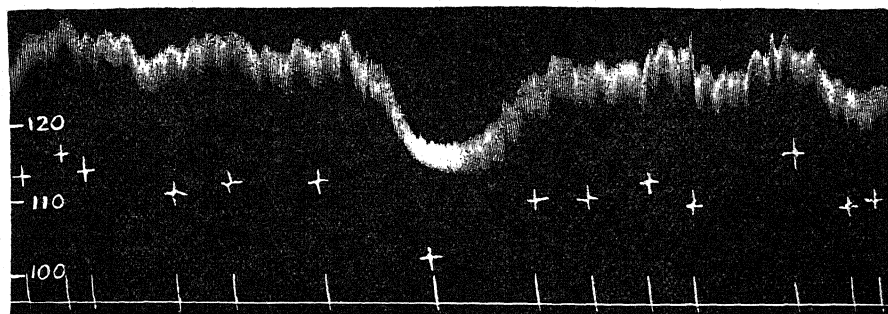


FIG. 3.—Effect of inhalation by S.G.M. of amyl nitrite on the sphygmomanometric oscillogram and on the sphygmomanometer readings.

to the added reflex times of observer and assistant are apparent, otherwise the variations in blood pressure run a close parallel. The instrument may therefore be used in a qualitative way for indicating rise and fall of blood pressure.

In the subjects investigated, inflation of the cuff to a pressure of 60 to 70 mm. of mercury was usually found sufficient to transmit good pulsations from the brachial artery; this pressure allowed a fairly free circulation in the deeper structures of the forearm, but completely obstructed the superficial veins. The immediate effect of the inflation was to cause a short initial drop, followed by a long-sustained rise of blood pressure, which gradually returned to normal in about 10 minutes (fig. 4). During this early period of venous filling the

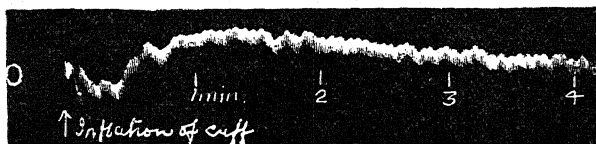


FIG. 4.—Rise of blood pressure following inflation of the cuff.

inflated cuff occasioned a little discomfort, but afterwards it was easily tolerated for half an hour or longer.

The right forearm was placed in a plethysmograph (fig. 1) made of a tin cylinder capped with a piece of 4-inch motor tyre and covered with a cotton-

wool jacket. It was filled with water at 35.5° C. and connected to a piston-recorder. This plethysmograph was also fitted with a quartz window.

The source of light used was an air-cooled quartz mercury-vapour lamp ($\frac{1}{2}$ kw.). Heat rays could be filtered off by means of a rectangular trough faced with quartz plates and filled with distilled water; most of the visible rays could be cut off by means of an adjustable cobalt quartz plate. The filter was attached to a rectangular window in a large asbestos shield. Between the filter and the lamp a removable asbestos screen was placed, which could be silently removed by a trained assistant. Disturbing factors were reduced to a minimum by attention to details contributing to the subject's comfort and by carrying out the observations in a dark room lit only by the stray light from the mercury lamp.

Controls.

The careful support of the subject's body in the chair and apparatus in a quiet, darkened room, with its attendant inhibition, revealed certain phenomena which are more particularly interesting in their relation to cerebral function. If the subject showed any tendency to fall asleep, the volume of the limb developed a series of long undulations superimposed on the small pulses and medium-sized respiratory excursions. When the room was placed in complete darkness the arm vessels exhibited a slow and large relaxation if the patient fell asleep, and showed a quicker return to normal on rousing the subject. It appears that the phenomenon of irradiation of cortical inhibition, which is the basis of sleep, also spreads to the sub-cortical centres. It became necessary, therefore, to maintain subjects in a more or less wakeful, yet relaxed, condition. Since the mere switching on of *any* light caused a slight vaso-constriction and small disturbances in the blood pressure, it was convenient to leave the mercury lamp in action continuously, the subject being protected by the large asbestos shield and removable screen already mentioned. This also eliminated any possible indirect effect of the mercury lamp on the air breathed, especially as Kestner (7) attributes a vaso-dilator action to substances formed in the neighbourhood of a carbon-arc lamp; it may be mentioned, however, that Kimmerle (8) did not find any appreciable action upon the composition of the air in the neighbourhood of a quartz mercury-vapour lamp. The latter, only, was used in this investigation.

Effect of Ultra-violet Radiations.

As the employment of filters involves a large diminution in the quantity of radiant energy—even in that part of the spectrum it is desired to transmit—

a highly polished nickel reflector in the form of a half-cylinder with spherical ends was used to concentrate as much light as possible on to the filter ; with this, nearly 50 per cent. more ultra-violet energy was available. The lamp was fixed at close range : 12 cm. in these experiments. In these circumstances an efficient fan was necessary for cooling the lamp and removing hot air from the neighbourhood of the subject.

As soon as the latter is fixed in the apparatus, with the asbestos screen in position, the lamp and fan are set into operation. After 10 to 20 minutes, the blood pressure and limb volume tracings assume a level or slightly undulating base-line. The asbestos screen is then silently removed, so as to expose the back of the subject to ultra-violet radiations. The normal dose was two minutes—an exposure sufficient to produce subsequently a smart erythema, and, still later, a brownly exfoliation and brown pigmentation.

The most characteristic circulatory response appeared in the records of the limb volume. The usual effect in a white subject is shown in fig. 5 ; the general

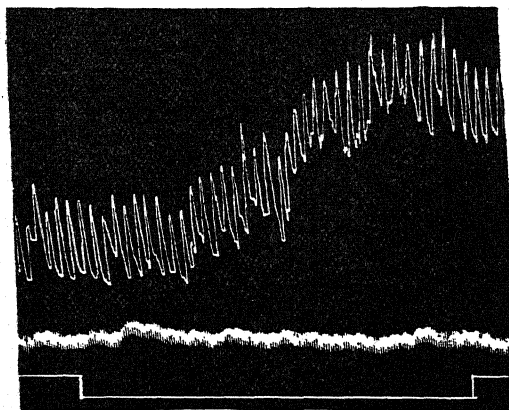


FIG. 5.—Response of a white subject (R.W.H.) to two minutes' ultra-violet radiation.

nature of the response is very similar in the dark-skinned individual (fig. 6), but is much less in degree for a given dose ; the latter characteristics appear in white subjects who have developed pigment as a result of previous exposures to ultra-violet radiations. In nearly all the cases examined, the limb responds with a marked vaso-dilation, which has a latent period on the average of $1\frac{1}{2}$ minutes, as a rule shorter in blondes and longer in brunettes ; then follows a slow and gradual return nearly to normal in two to five minutes. It is not surprising that the observed increase in pulse rate of from two per minute in dark subjects to five per minute in white subjects, during irradiation, is not

accompanied by any great rise in the blood pressure in view of this very efficient compensatory generalised vascular dilatation. In most white subjects, however,

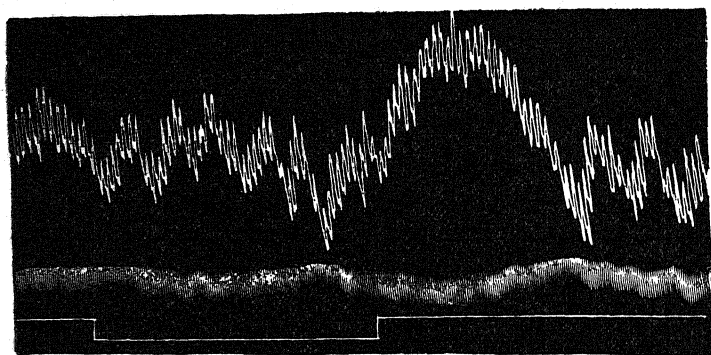


FIG. 6.—Response of a pigmented subject (Bombay) to $1\frac{1}{2}$ minutes' ultra-violet radiation.

the most usual blood pressure response was a very slight rise during irradiation, followed by a similar slight fall and gradual return to normal. Hasselbalch (9) was the first to record any rise of blood pressure during irradiation, his measurements giving values as large as 5 to 10 mm. of mercury; but such figures were obtained by employing the total radiations from a carbon-arc lamp. It may be noted that the graphical method used in the present investigation constantly manifests in all subjects a rise of blood pressure immediately following inflation of the cuff of the sphygmomanometer (fig. 4); it is possible that part of the rise noted by Hasselbalch may have been due to this.

Of the forty medical students repeatedly examined, the normal response was the vaso-dilatation indicated above in figs. 5 and 6, and was repeatedly elicited in the majority of them. A few (five) consistently gave an appreciable preliminary vaso-constrictor response as shown in fig. 7. This difference in response appears to be merely a climatic effect, since this larger initial vaso-constriction was obtained regularly on the very hot days in the beginning of June of this year and showed greater persistency in sultry weather; presumably on these days, the blood vessels were already nearly dilated to the maximum before the experiment started.

In an attempt to determine what events were taking place at the actual site irradiated, a small group of mixed subjects was selected for their steadiness under examination, so that delicate plethysmograph tracings with high magnification might be made, while a small part of the limb itself was irradiated through the quartz window (fig. 1). The arm was supported with a wool pad

placed in the plethysmograph quite near to the quartz window, so that it became possible to irradiate the skin on the forearm at the short range of 8 cm. With

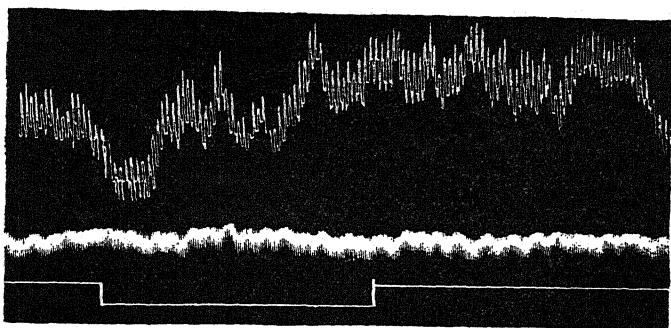


FIG. 7.—Response of R.W.H. to ultra-violet radiation (2 minutes) on a hot, sultry day.

the exception of the writer's, all the tracings showed an initial vaso-constriction, which, in the case of short exposures, was followed by a return to normal (fig. 8).

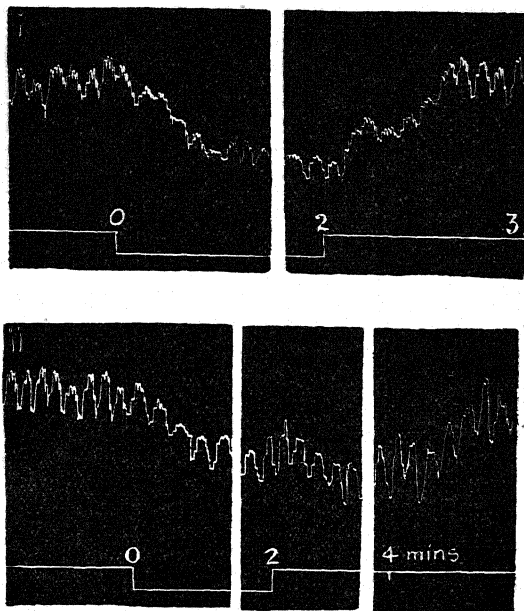


FIG. 8.—Irradiation of limb in plethysmograph. Two consecutive 2-minute exposures of A.H. to ultra-violet radiation.

The phenomenon of summation of stimuli also was exhibited in this local response, as can be seen in the lower curve in fig. 8, which shows a long after-discharge.

A survey of the local and distant vascular responses enumerated above, together with the existence of a smarting or tingling sensation (compare sunburn) referred to the site irradiated, suggest that the mechanism by which the radiations work is a nervous one. The high absorptive coefficient of the epidermis for ultra-violet radiations must involve the degradation of this type of radiant energy into heat, with consequent excitation of the heat receptors in the skin; the matter, however, is not quite as simple as this. Corresponding to the difference in the sensations produced, a smarting and tingling element in the case of ultra-violet and warmth in the case of infra-red rays, there is also a difference in the circulatory response to ultra-violet and to heat radiations, which will be presented subsequently.

In some cases slight alterations in the respiratory rhythm were noted, but these were inconstant; examples are presented in fig. 9. Periodic breathing was a frequent occurrence.

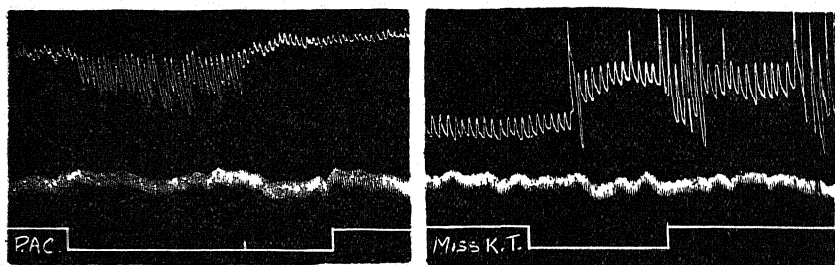


FIG. 9.—Effect on the respiratory rhythm of $1\frac{1}{2}$ minutes' ultra-violet radiation.

Effect of Two Successive Irradiations.

In the following, as in the previous experiments, the skin of the back was chosen for irradiation because the irritation in the erythematous stage was less troublesome than on other parts of the body, and, further, the manipulations were not in the view of the subject. At each exposure, 100 sq. cm. of skin were subjected to the filtered rays of the quartz mercury lamp. In any given subject the general responses due to irradiation of the left and the right scapular regions separately were precisely equal. When, however, a second irradiation was applied to the same area within a few minutes after the first exposure, the response assumed new characters: the latent period was considerably shortened and the extent of the vaso-dilation was much increased (fig. 10). The equality of these distant and general responses elicited from equal stimulation of different areas of skin show that there is no change of a central nature induced by the first exposure, whereas comparison of records like those of fig. 10 shows that

some kind of after-effect persists in the periphery as a result of a previous stimulus which raises the excitability of the receptors involved, giving an

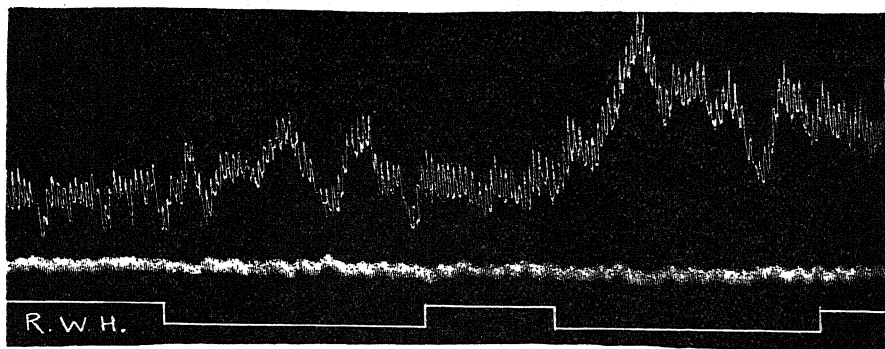


FIG. 10.—Summation of stimuli: two 2-minute exposures of the same area to ultra-violet radiation with a 1-minute interval.

exaggerated response for an equal exposure. As far as temporary applications of the apparatus to the subject permitted a deduction to be drawn, the after-effect seemed to last as long as the erythema.

It appears that the lowering of the threshold of these particular receptors is an effect characteristic of radiant energy of short wave-length. For the examination of the effect produced by longer wave-lengths, the radiant energy reflected by a nickelled parabolic reflector from a spiral coil of nickel-chrome wire situated at the focus was projected on to the subject's back. The energy consumption of this electric heater was half a kilowatt; the heater was placed a metre away from the subject, this being the distance at which the rays were well focussed. The application of successive irradiations of this type usually yielded an equal or smaller response (fig. 11); in none of the subjects examined was a larger response evoked. It appears, therefore, that the sensory end-organs affected here—the heat receptors—exhibit the usual phenomenon of fatigue on repeated excitation.

The summation of ultra-violet stimuli found to occur in these experiments of short duration is in complete accord with personal experience of successive daily exposures of the same area to sun burning. Side by side with the sensations of tingling and smarting, we have the fundamental observations of Finsen (10) who, in his experiments with a 40,000-c.p. arc lamp, noted that the increased excitability of the capillary wall as tested by mechanical stimuli persisted for over six months. As a provisional hypothesis for explaining the mode of action of ultra-violet radiation, it is suggested that it

behaves as a cutaneous irritant, manifesting itself by sensory and vascular phenomena involving an axon reflex (11), and also a distance reflex; this supposition opens up problems for further investigation.

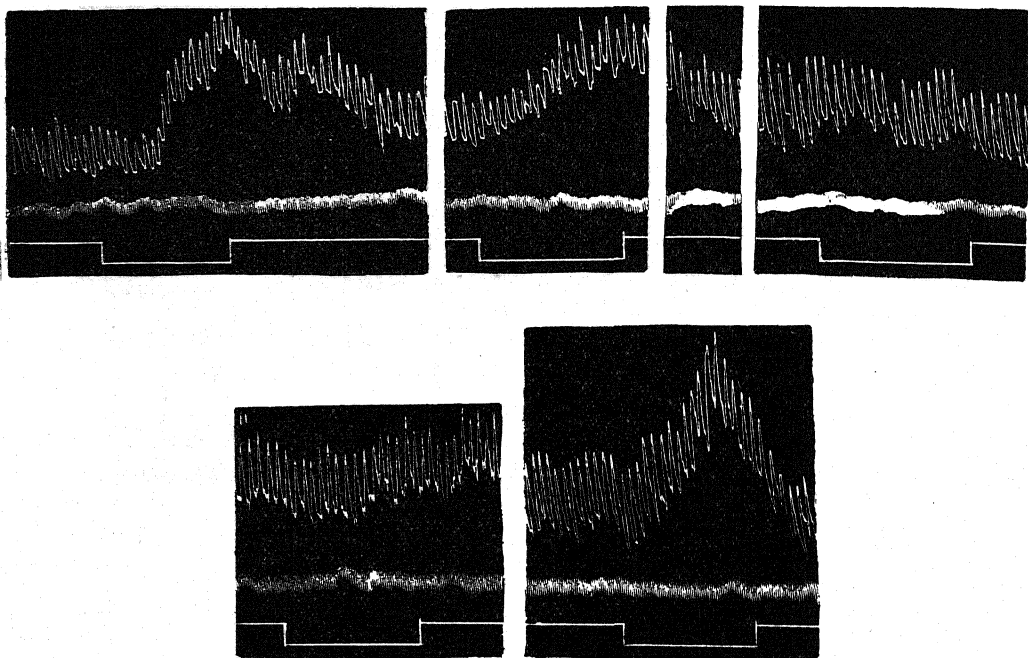


FIG. 11.—Effect of successive stimuli (1-minute exposures) on Miss K.T. : (i) Radiant heat
(ii) Ultra-violet radiation.

Effect of Intense Stimulation of Anaesthetised Animals.

Cats were used in these experiments, and were placed under the action of chloralose. The fur was removed from an area of 100 sq. cm. on the animal's back, and the skin depilated with barium sulphide. An arterial cannula was placed in the carotid artery; the hind limb was placed in a plethysmograph and firmly attached thereto by reflecting a cuff of skin and tying it over the mouth of the plethysmograph. Irradiation was carried out as before, but at the closest possible range (7 cm.) and for much longer periods.

As a rule, the limb volume followed the blood pressure more or less completely—a result due in all probability to the removal of the sensory element by anaesthesia. Responses, always of a pressor type, could be evoked in albino cats only after prolonged or frequent stimulation with ultra-violet radiations. It was even more difficult to elicit as large a response in an all-black cat. In order to make a better controlled comparison, a parti-coloured

cat was prepared with a white and a black patch for irradiation. The same difference was exhibited, namely, a greater susceptibility of the white patch. The part played by pigment in the skin of animals has also been examined in an earlier paper (12).

When the unfiltered radiations of the mercury arc were used, a response was obtained precisely like that from radiant heat. Cutting both vagus nerves in the neck caused no difference in the response (fig. 12).

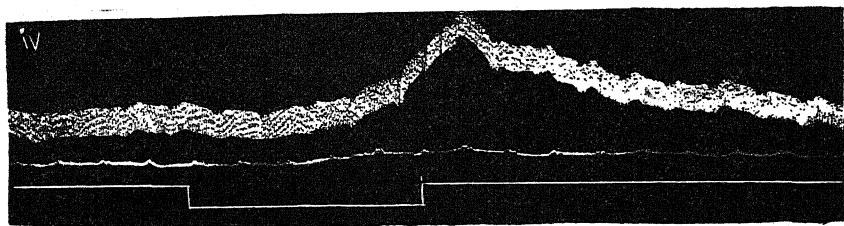


FIG. 12.—Response of a white cat (vagi cut) to $1\frac{1}{2}$ minutes' radiant heat.

The long latent period and the simple pressor character of the response suggest that the basis of all these reactions is stimulation by heat only. Identical responses were also obtained in an aged grey cat, with a blood pressure of 190 mm. mercury, even when this was temporarily raised artificially by obstructing the cerebral circulation. The chief result of these animal experiments is to show that massive nocuous doses are necessary to produce appreciable differences in the blood pressure during anaesthesia.

Summary.

Irradiation of a localised area of skin with ultra-violet energy causes a wide-spread peripheral vaso-dilation. Consequently, only a very slight transitory rise of blood pressure results from the small increase in pulse rate of from two to five per minute in dark and white subjects respectively. The reaction was always less in pigmented subjects.

The vaso-dilator response to ultra-violet radiation is enhanced by a previous exposure; this is not the case with radiant heat. The vascular response appears to be a nervous reflex, initiated by a nocuous stimulus, and the whole phenomenon one of incipient injury.

REFERENCES.

- (1) Hasselbalch and Jacobäus, 'Berlin. kl. W.' No. 39 (1907).
- (2) Bach, H., 'Deutsche Med. Woch.', No. 9 (1922).
- (3) Königsfeld, H., 'Zeitschr. f. kl. Med.', vol. 91, p. 159 (1921).

- (4) Sequeira and O'Donovan, 'Lancet,' I., vol. 18, p. 911 (1925).
- (5) Rollier, A., 'Heliotherapy,' Oxf. Med. Pub., 211 (1923).
- (6) Kolls, A. C., 'Journ. Pharm. and Exp. Therap.,' 15, vol. 5, p. 443 (1920).
- (7) Kestner, O., 'Zeitschr. f. Biol.,' vol. 73, p. 7 (1921).
- (8) Kimmerle, 'Strahlentherapie,' vol. 13, p. 290 (1921).
- (9) Hasselbalch, 'Skand. Arch. f. Phys.,' vol. 17, p. 431 (1905).
- (10) Finsen, *see* Krogh's 'Capillaries,' p. 103.
- (11) Bayliss, W. M., 'Jour. Physiol.,' vol. 26, p. 173 (1901).
- (12) Harris, D. T., 'Proc. Roy. Soc.,' B., vol. 98, p. 178 (1925).

The Mechanism of Muscular Contraction.

By W. E. GARNER, Reader in Physical Chemistry at University College,
London.

(Communicated by Prof. A. V. Hill, F.R.S.—Received August 6, 1925.)

A contracting muscle gives maximal values for the force exerted only when it is allowed to shorten very slowly. The force exerted decreases as the speed of shortening increases.

The nature of the physical property which underlies the contractile mechanism, and which is responsible for the variation of force exerted with speed of shortening, is not definitely known. It may be any of the properties of heterogeneous systems which attain equilibrium slowly, such as the properties of viscous elastic systems, the generation of interfacial tension, etc. The properties of viscous elastic systems have already been applied to the problem of a muscle shortening at varying speeds, and as Gasser and A. V. Hill (1) have shown, equilibrium is established in a viscous elastic system under stress at rates which show many analogies with those observed during the contraction of muscle.

On any surface theory of muscular contraction, another property of heterogeneous systems, viz., the slowness of diffusion of adsorbed substances away from the interface, may be invoked to explain the slow rate of attainment of an equilibrium value of the force exerted. The interfaces between the different phases undergo marked decreases in area during contraction, and these changes must entail increases in concentration in the interfacial layers. These concentrations may be much higher than the equilibrium values corresponding to the new length of the muscle, and since the establishment of equi-

brium by diffusion is a comparatively slow process, there will be a lag in the development of the equilibrium interfacial tension. This lag will be the greater the more rapid the shortening.

Such theories of the cause of the decrease in the mechanical output of a muscle under increasing speeds of shortening are not alone sufficient to explain all the phenomena observed during shortening. For example, when a muscle undergoing a maximal isometric tetanus is suddenly released from a longer to a shorter length, the tension always falls below the isometric tension at the shorter length (2). If the decrease in the length is of the order of 10 to 15 per cent., the whole of the tension disappears momentarily on release. The tension corresponding to the shorter length is then developed from zero, at a speed which shows that the property of the muscle producing the tension has been completely destroyed by the release. On the other hand, the effects of extension of a stimulated muscle are not so destructive of the force exerted. This never falls to zero on rapid extension from one length to another.

The shattering effect of a sudden release calls to mind the experiments of Langmuir and of Adam (3) on the behaviour of thin films of aliphatic monobasic acids on water. The films of the higher acids become solid under compression, and when subjected to a force of 60 dynes per cm. begin to break down. The surface, invisible at lower compressions, begins to crumple, and visible ridges and lines are formed on it. The film is now no longer uniform nor continuous, and on account of the slow rate of molecular diffusion in the solid state, the recovery of uniformity and continuity is very slow. Thus, if the tension of a muscle fibre be produced by the formation of a solid monomolecular film on the surface of the fibrils, the disarrangement of this film, which must occur on sudden release, would momentarily reduce the tension to zero. The tension would then be re-developed by the repair or rearrangement of this solid film. The rate of repair would be similar to the initial rate of formation of the film. Thus, the assumption of the formation of a solid film on the surface of the fibrils during excitation may quite well account for the relations established between the speed of shortening and the work done.*

An examination of the physical and chemical properties of muscle reveals

* A muscle fibre, however, possesses a complex structure and the solid film may not cover the whole surface of the fibrils. It may be found only on the anisotropic zones, in which case the release may not immediately be followed by a disarrangement of the solid film. The decrease in surface which results from shortening may first occur on the isotropic regions of the film and subsequently be extended to the anisotropic zones. A sudden release followed by a quick stretch may thus have little effect on the tension.

several cogent arguments in favour of the above view. One may mention, for example, the marked increase in viscosity and elasticity of muscle on excitation (1), which is readily understandable if a solid film is produced on stimulation.

In the following pages an attempt has been made to collect together the evidence bearing on the possible existence of this solid film and, assuming that it is present, to make deductions as to the part which it plays in the contractile mechanism.

Molecular Orientation in the Muscle Fibre.

The occurrence of some form of molecular orientation in muscle has long been known. Beck, in 1839, discovered that striated muscle was doubly refracting. Subsequent work on dead and living muscle has confirmed the observation and has shown that the optical anisotropy is limited to well-defined segments of the muscle. A muscle fibre appears in longitudinal cross-section as if it were constructed of fibrils, each of which is regularly segmented, the separate layers exhibiting marked differences in their optical and other physical properties (4). One of the regularly repeated segments is very strongly positively doubly refracting. Its chemical nature is unknown, but it behaves like a protein in that it swells in dilute acids. The optic axis usually lies in the direction of the length of the fibrils. During the contraction of the muscle, the anisotropic and isotropic striæ or rods undergo a rapid shortening; the material composing the segments undergoes considerable movement under pressure. The anisotropic regions thus possess the mobility and structure normally associated with crystalline liquids. Under the movement, the anisotropy decreases, a change expected if the anisotropy be due to the liquid crystalline state of matter. There has been much discussion as to the nature of the volume changes on contraction, but as most of the observations have been made on dead muscle (5), caution must be exercised in applying the conclusions reached to living muscle. Hürthle (6), however, maintains that the isotropic portions of the living muscle increase in volume at the expense of the remaining segments.

The chemical nature of these anisotropic bodies is uncertain. They very probably consist of compounds with long carbon chains, since anisotropy in the liquid state is almost exclusively limited to this type of compound. The lateral attraction between the carbon chains forms an essential element in the stability of the anisotropic structure. Since several naturally occurring lipoids, cerebrosides, and cholesteryl derivatives, *e.g.*, lecithin (7), kephalin (8),

phrenosin (9), protagon (10), etc., are known to occur in the anisotropic liquid condition, one or other of these substances may be present in the anisotropic segments. It is unlikely that they form the whole of the non-aqueous material in these segments, since these behave towards acids as if they contained protein. Indeed, the possibility must not be overlooked that the protein itself occurs in the liquid crystalline state.*

Conclusions of a general character may, however, be drawn as to the nature of the orientation in the muscle fibrils, from observations made on the structure of anisotropic liquids and the solids of the long-chain carbon compounds. Microscopical and X-ray examination of anisotropic liquids has thrown light on the nature of the molecular orientation. It is very much less complete than in solid crystals. Types have been discovered ranging from those which show no structure under X-rays to those of which the X-ray spectrogram is scarcely distinguishable from that of solid substances. G. Friedel (11) has classified them into nematic, cholesteryl and smectic groups respectively. Up to the present, X-ray examination of the first two groups has failed to reveal the exact nature of the molecular orientation (12), but from microscopic examination Friedel deduces that the molecules are arranged in chains normal to the bounding surfaces of the liquid. In the cholesteryl, but not in the nematic type, a spiral arrangement of the molecules is present. Anisotropic liquids of the smectic type, under the microscope, show a focal structure, *liquide à conique*. On examination with X-rays, they show a set of equidistant planes along which the molecules are arranged. Ethyl *p* azoxy-benzoate and ethyl *p* azoxy-cinnamate, which are definitely *liquide à conique*, possess one set of such planes (13), the distance between which is greater than between corresponding planes in the solid state. Soap curds, the oleates of ammonium, sodium and potassium, show evidence of orientation in three directions in space (14). Their degree of molecular orientation and their rigidity are intermediate in character between those of the smectic and the solid states. Piper and Grindley (15), from an X-ray examination of soap curds, came to the conclusion that the carbon chains are arranged as in crystals of the aliphatic acids (16). In these the crystal unit contains two chemical molecules with the carboxyl groups in juxtaposition. The juxtaposed carboxyl groups lie in sets of parallel planes throughout

* The results of Stübel ('Archiv für Physiol.,' vol. 201, p. 643 (1923)) indicate that the double refraction of muscle fibre is due to the presence of negatively double refracting lipid, and positively double refracting protein. In addition, a double refraction is present due to a rod-like arrangement of the protein particles.

the crystal, and the terminal methyl groups lie in planes parallel to these.

In the solid state the inclination of the carbon chains to the planes containing the terminal groups varies with the nature of the terminal group. The direction and intensity of attraction between these groups determines to some extent the direction of the carbon chains. In the esters of the aliphatic acids, the chain direction is at right angles to the planes *ab* (fig. 1) containing

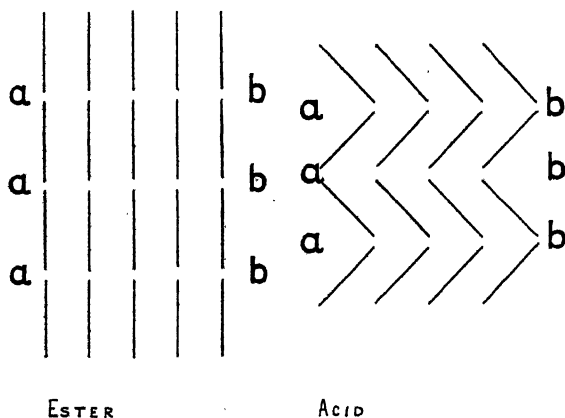


FIG. 1.

the terminal groups (16), but in the aliphatic acids the angle is approximately 55° .* From the nature of the figures obtained under the microscope with convergent polarised light, the structure for these acids would appear to be that shown in the figure (17).

If the anisotropic segments in a muscle fibre be composed of substances in the smectic state, *e.g.*, of substances like lecithin, protagon, cholesteryl stearate, salts of fatty acids, etc., or even the sodium salts of proteins, the molecular orientation will very probably resemble that of the esters of the fatty acids. The chains of stearic, linoleic acids, etc., will be orientated in a direction parallel to the optic axis (14), *i.e.*, to the axis of the muscle fibril in striated muscle. The sets of planes containing the terminal groups will be at right angles to this direction. The viscosity of the structure may be somewhat high.

If the anisotropic liquids are nematic or cholesteryl in type, *e.g.*,

* Müller ('Nature,' vol. 116, p. 45 (1925)) has recently shown that the angle for one form of stearic acid is 59.7° .

cholesteryl butyrate, the chains of molecules will be in the same direction, but the viscosity will be much lower. The arrangement of the terminal groups in sets of planes may be absent. This reduces the viscosity to such an extent that the Brownian movement of dust particles across the crystal boundaries may be unhindered (11).

Formation of Solid Films and the Production of a Tension.

The source of the energy of muscular contraction is the free energy of the change glycogen to sodium (or potassium) lactate, and in the recovery process the conversion of a portion of the latter into carbon dioxide. The mechanism by which these changes are brought about, whether electrochemical or chemical, is not material to the present argument. An essential step in the process is, however, the production of lactic acid. This substance, a moderately strong acid, $K = 1.38 \times 10^{-4}$, almost fully ionised at the pH of the interior of the muscle, might by processes of hydrolysis bring about changes in the materials composing the segments of the fibrils. The esters and salts of the fatty acids, lipoids, etc., present in the anisotropic segments, might, therefore, be converted into fatty acids which, if produced in the surface molecular layer of the segments, would be capable of giving solid films. Several reasons may be advanced to show that this change would be accompanied by the development of a tension.

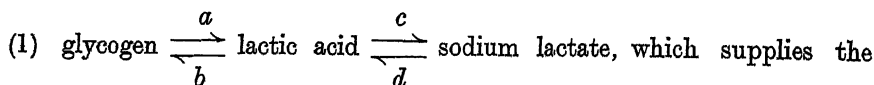
(1) The anisotropic liquids possess a looser structure than the solid state. The distance between the sets of planes is greater (13). The change of a film of a crystalline liquid into the solid state would occur with contraction in the direction of the optic axis.

(2) A chemical change occurring at the terminal groups, *e.g.*, a removal of phosphoric acid complex from lipoids, of alcohol from ester, and the conversion of a salt into an acid, would result in a decrease in the length of the unit crystal cell.

(3) The change in the chemical nature of the terminal group may cause re-orientation of the carbon chains as in fig. 1. This would produce a shortening in a direction parallel to the axis of the muscle fibril. These effects, occurring either singly or together, might account for the rise of tension occurring in the initial stages of the activity of a muscle fibre.

The Source of Potential Energy in Muscle Contraction.

The precise nature of the excitation process, and the detailed mechanism of the consequent chemical reactions, are unknown. The main chemical change, however, is the conversion of glycogen into sodium lactate,



chemical energy set free in the initial phases of the contraction.

In the resting muscle, glycogen is only very slowly converted into lactic acid, and the reverse change (b) occurs but slowly in the recovery phase, when it is accompanied by the oxidation of part of the lactic acid to carbon dioxide. Both of the processes appear to be speeded up in stimulated muscle. There is no doubt about the increase in speed of conversion of glycogen into lactic acid during stimulation, but there is only indirect evidence for the speeding up of the reverse change (*see* p. 54).

Many of the phenomena occurring during muscular contraction may be accounted for if it be assumed that the production of lactic acid follows a reversible reaction of the type :—

(2) $A \rightleftharpoons B \rightleftharpoons \text{lactic acid}$, which occurs in the resting muscle very slowly. A is a product intermediate between glycogen and lactic acid, possibly a hexose phosphate, which is present in small amount and replenished from the reserve of glycogen as it is itself used up. B is an active intermediate form of both A and lactic acid, which possesses a short-life period. It is assumed that it is formed from both A and lactic acid with an absorption of energy, and hence can only be produced from molecules with energies higher than the average. The number of B giving A bears a fixed ratio to the number giving lactic acid, otherwise a position of equilibrium in the system could not be established. The velocity of approach towards equilibrium is dependent on the rate of production of B. This proceeds extremely slowly in the resting muscle, but is catalysed by some unknown mechanism during the process of excitation of the muscle.

Molecular Activation by Surface Energy.

Leaving on one side the mechanism by which A is activated on stimulation which it may be possible to explain on the Lillie model (18), it is of interest to consider a general case, the possibility of activation as a direct result of the changes in surface energy during the movement of the interfaces of the muscle

fibre. This is a factor which may play a large part in the economy of the living cell.

In a moving heterogeneous system such as living muscle, where the area of the surfaces is continually changing, there will be fluctuations in the surface energy which will lead to local changes in the energy content of the molecules adjacent to the surface. These changes may play a part in speeding up or retarding chemical change. For example, energy is liberated on decrease in area of a surface. As the surface decreases, since the energy of the surface molecules is higher than that of the average molecule in the interior of the liquid, some of these molecules will pass into the interior with energies above the average, and possibly their energy content may be sufficiently high to enable them to undergo chemical change. On the other hand, a portion of their energy may be dissipated as heat to their surroundings. In the case of an orientated surface film, it is possible that all of the molecules liberated by the decrease in area undergo chemical change.

The same arguments will apply to the change in area of a surface which is generating a tension, or to a change in the tension of a surface at constant area. In these cases still larger amounts of energy may be available for molecular activation. Under favourable circumstances, it would be possible for a large proportion of the free energy change of a surface, not utilised as external work, to be stored in this way as chemical energy. Thus if, owing to any cause, the whole of the energy of a surface is not utilised in doing external work, it might be stored as chemical energy and as such again available as a source of potential energy. Thus in the muscle a mechanism may be included by which part at least of the energy set free and not employed in doing external work may be stored as chemical energy. In terms of the chemical changes occurring in the muscle, this means that when the energy liberated during the conversion of glycogen to lactic acid is not utilised in doing external work, in part at least, the conversion of lactic acid back into glycogen might occur.

A Model of Muscular Action.

A model of a segment of a muscle fibre is put forward in this paper, which it is hoped may illustrate the nature of the variations in the potential energy and tension obtained on stimulating a muscle fibre under various conditions. In fig. 2, a longitudinal section is given of one of the anisotropic segments of a muscle fibril. The section *cd d'c'* is supposed to consist of an anisotropic liquid in the form of a potassium salt, and to be permeated by alkali. Lactic acid is

assumed to be produced on membrane I inside the sections $abdc$ and $a'b'd'c'$. It is thus contained within the two membranes I and II, which are regarded as

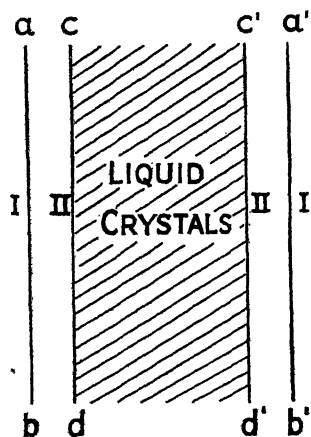


FIG. 2.

being a few molecular layers apart. The lactic acid produced reacts on the surface of the anisotropic liquid to give a solid membrane, with the formation of potassium lactate. This is imagined to produce a tension parallel to the axis of the fibril. On relaxation, the main change is the disappearance of the solid film under the action of alkali. Thus a series of consecutive reactions* occurs in the form of a pulse across the membranes into the interior of the fibril. Movement of the membranes while the pulse is in progress will induce energy changes in the path of the reactions. The effect of such changes will obviously depend on the

time which has elapsed after stimulation. These phenomena will be dealt with later.

Production of Energy in Muscle.

The amount of energy set free in a muscle fibre is dependent not only on the duration of the stimulus, but also on the temperature and the mechanical conditions obtaining during contraction (19). The series of chemical and physical processes set in motion by the stimulus are affected by changes occurring in the muscle subsequent to the initiation of the pulse. The changes commencing with the disappearance of glycogen, and terminating with the formation of sodium lactate and the liberation of heat and mechanical energy, are capable of being accelerated, retarded, or even reversed, by stretching or shortening the muscle at various stages after stimulation. The correlation of these numerous factors is a matter of considerable difficulty, especially as information about the mechanical structure and chemical composition of the muscle is very scanty. It is thus impossible to do more than to suggest a correlation of a general and somewhat tentative nature. It is hoped that this will be possible with the aid of the conceptions advanced above.

The simplest case is that in which the muscle is stimulated at the normal resting length under such conditions that the length does not vary during

* There should be no difficulty in dealing with these reactions quantitatively, providing the tension-time curve gives a true measure of the state of the solid film.

the activity. It is under these conditions that the simplest relationships have been found between the tension and the energy liberated.

The duration of and the total energy liberated in an isometric twitch decrease with increase in temperature. A. V. Hill (20) has suggested that the shorter duration of the twitch is the cause of the smaller evolution of energy. This suggestion appears to give a satisfactory explanation of the facts without providing a detailed mechanism for the operations by which the conversion of glycogen is initiated. It is clear that the amount of chemical change at a membrane must be proportional to the time that the membrane is in operation.

In a prolonged tetanus the energy consumed in maintaining the tension may be larger than that required initially to establish it. The rate of development of energy here becomes an important factor. This is governed (*a*) by the rate at which glycogen can be converted into its activated form, and (*b*) by the rate at which the substance generating the tension is removed from its sphere of action by alkali. A stationary state is induced, as long as the tetanus lasts, in which the rate of flow of energy through the system is governed by the rates of the processes occurring at membranes I and II (fig. 2). At I the principal process is the conversion of glycogen to lactic acid, and at II the formation of the solid film and its destruction by alkali. The rates at which the processes take place at the two membranes will very probably be dependent on one another. Certainly the rate of formation of the solid film will be determined by the rate of liberation of lactic acid at membrane I. It is conceivable also that the rate of production of lactic acid at I may not be independent of its rate of removal at II. An accumulation of lactic acid between the two membranes may set back the velocity with which glycogen is converted into lactic acid.

Any changes at membranes I and II will of necessity affect the rates of reaction and the rate of flow of energy through the system. These changes at the membranes may be of two types. They may be such that changes in area of the membranes are made and completed *before* excitation, as is for all practical purposes the case in measurements of isometric tension on muscle at varying lengths; or the membranes may be disturbed by movements, extension or shortening, *during* the period in which the train of chemical and physical changes passes across the muscle fibre.

Variation of Total Energy with Length of Muscle.

The variation of total energy with length has recently been investigated by A. V. Hill (21) for lengths both greater and less than the normal resting length.

The results are shown in fig. 3 (p. 53), which is self-explanatory. A maximum heat evolution occurs at or near the resting length. In these experiments, since the stimulus may be of short duration, the heat evolution is very largely determined by the events happening at membrane I. We can leave out of account those at membrane II.

In order to obtain an insight into the relationship between length of muscle and heat production, a knowledge of the mechanism occurring at membrane I is needed. This is lacking, but a qualitative explanation of the results may be given if it be assumed that the transmission of the stimulus occurs along a monomolecular film which is situated on membrane I. The rate of production of lactic acid may be proportional to the area of this film and its state of compression. If it be assumed that this monomolecular film covers the surface of the fibre completely at the resting length, then a qualitative explanation of the maximum may be given in terms of the change in properties of this film when extended or shortened. On extending the film it will become discontinuous, and this change, possibly by modifying the transmission wave, may result in a decrease in the heat production for lengths of muscle greater than the normal resting length. On shortening, the film will be compressed and its area reduced as \sqrt{l} , and this may account for the decrease in the heat liberated on shortening the fibre.

In a prolonged tetanus the area of the second membrane and its effect on the rate of formation of sodium lactate will very probably have to be taken into account. This rate would appear to increase with the area.

The effect of movements of the membranes during the development of a tension would be expected to give rise to a complex group of phenomena in which the changes in surface energy of the membranes will play an important part. These will be discussed later.

Development of Tension.

(a) *Isometric twitch at the resting length of the muscle.*—In a muscle twitch, stimulated by an induction shock or a tetanus of short duration, a constant relation exists between the heat production and the tension developed (20). H/Tl is a constant independent of the temperature, for a muscle at lengths not very different from its resting unloaded length. H is the heat production in ergs, T the force in dynes, and l is the length of the muscle in centimetres. With these units, $H/Tl = 0.21$. This ratio is all the more important since although temperature affects both H and T it leaves the ratio constant.

In terms of the chemical changes occurring in muscle, this relationship can

be interpreted only in one way, namely, that the force developed is proportional to the amount of glycogen converted into sodium lactate. This relationship between the force developed and the sodium lactate produced receives a simple interpretation on the above model. The sodium lactate is regarded as being produced only by reaction between lactic acid and the anisotropic liquid surface. This reaction is imagined to set free one straight-chain acid for every molecule of lactic acid liberated. The force developed is thus proportional to the number of molecules of straight-chain acids set free on the surface of the fibrils. The force generated may be written $T = kmnr$, where T is the force, k is a constant, n the number of molecules of lactic acid liberated on unit area, r the radius, and m the number of fibrils in the muscle.

(b) *Prolonged tetanus*.—There are two components in prolonged tetanus, one concerned with the development of potential energy and the other with its maintenance at a constant level. The energy required in the two processes can be represented by the equation, $H = Tla(l + bx)$ (20) in which H is the total energy, T is the maximum force, l is the length of muscle, x is the duration of stimulus, and a and b are constants: a is independent of temperature, and b increases 2.3 times for 10° . It is suggested that Tla represents the chemical energy necessary to build up a solid film one molecule thick on the anisotropic segments, and that the term $Tlabx$ is the energy required to maintain the film so produced, which is attacked by alkali at the same time as it is being built up under the action of lactic acid. The temperature coefficient of b is that of the reaction between the solid film and alkali. This hypothesis of the formation of a monomolecular film could be readily tested by a simple calculation were the experimental data available. Approximate data already known serve to show that there is nothing inherently improbable in the assumption.

According to the calculations of A. V. Hill (2) the area of the muscle fibrils in 1 c.c. of muscle is 20,000 square centimetres (Bernstein) and the lactic acid liberated in a maximal contraction, if spread out as a continuous monomolecular film, would occupy an area of $1/44$ of this. This calculation was made on the assumption that the area occupied by one molecule of lactic acid is 21×10^{-16} square centimetres. The area occupied by a molecule of stearic acid, if it be arranged with the carbon chain in the surface, will be $l \times t$, where $l = 20 \times 10^{-8}$ centimetres, and $t = 4 \times 10^{-8}$ centimetres approximately. A monomolecular film of stearic acid could thus be produced, as a result of the chemical change occurring in a maximal contraction, on 2,000 square centimetres of muscle. This area is one-tenth of that of the muscle fibrils.

Since the area of the anisotropic segments is a greater fraction than one-tenth

of the total area, this amount of stearic acid is apparently insufficient to form a monomolecular film over these segments. Although stearic acid is actually present in the muscle, and would be a likely source of the solid film, it is possible that another substance, *e.g.*, a protein molecule, with an area about four times that of stearic acid, forms the contractile film. The calculation, however, of the area of the muscle fibrils is only approximate and the discrepancy may very probably be due to this cause.

An argument in favour of the view that the contractile mechanism is associated with the surface of the fibres, rather than that of the fibrils, is that sufficient lactic acid is produced in a maximal contraction to form a monomolecular film of this substance on the surface of the fibres. The area of cross-section of a stearic acid molecule is approximately the same as that of lactic acid, and a film of stearic acid arranged with its carbon chains at right angles to the surface contains approximately the same number of molecules as a monomolecular film of lactic acid. The optical evidence is, however, against the presence of a film in which the carbon chains lie at right angles to the direction of the fibre, for the optic axis of the anisotropic segments lies parallel to this length. It is a reasonable assumption to make that the orientation of the long chains in the solid film will be the same as in the anisotropic liquids forming the segments.

Length of Muscle and the Tension developed on Isometric Contraction.

The curve (fig. 3) showing the variation of tension with muscle length possesses points of similarity with the curve of heat production, giving a maximum nearly at the same point (21). The tension, however, decreases to zero when the length is reduced to one-half of the resting length of the muscle and would appear to decrease equally rapidly for the same percentage of stretching. The tensions on the right of the maximum are those due to the contractile mechanism alone, since the tension due to the initial stretching has been deducted. Those on the left, however, include the restoring force of the contracted muscle, which acts against the tension set up by the contractile mechanism.

These experimental results on the relationship between length and tension developed are difficult to explain in terms of the proposed model, mainly because of the complex nature of the phenomena. The change in length has affected not only the contractile mechanism but also the mechanism which regulates the initial conversion of glycogen into lactic acid. As the latter is not thoroughly understood, it is almost impossible to throw much light on the

above results. Also the manner in which the contractile mechanism is affected by extension or stretching is by no means clear. For lengths less than the

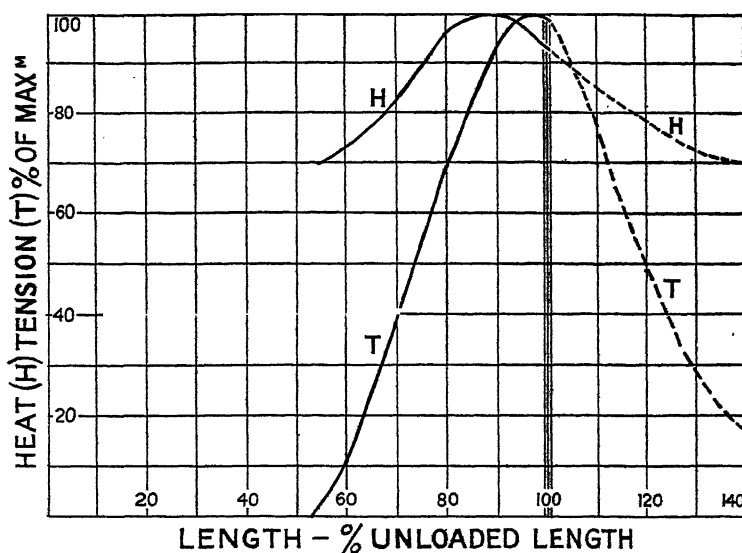


FIG. 3.

normal resting length, the relationship between the restoring force and length is unknown, so that the actual tension due to the solid film cannot be calculated. For lengths above the normal resting length, the decrease in tension may be due to the partial destruction of the surfaces of the anisotropic segments.

Potential Energy Changes in Muscle as a Result of Movement of the Membranes either During or After Stimulus.

A movement of the membranes while the reaction pulse is in progress across the fibrils will, it is anticipated, modify the speeds of the chemical reactions at the membranes. In the first place, compression and rarefaction of the membranes I and II will affect the speed. From analogy with gaseous reactions, where an increase in pressure affects the speed of bi- and multi-molecular reactions, it is probable that compression of a film or membrane will increase, and extension diminish, the velocity of the chemical processes occurring in it. In the second place, the liberation or absorption of energy by surfaces during the shortening or extension of the films will also influence the speed. This factor will operate in the same direction as compression and rarefaction.

During the stimulus itself the predominant change is the formation of lactic acid from the precursor A on membrane I, and this reaction will be accelerated

during the shortening of the muscle fibril and diminished during extension. Movement after stimulation has ceased will mainly modify the speeds of the chemical reactions occurring on membrane II. At this phase, a store of activated lactic acid will still be present between the two membranes. This store is undergoing change, partly into potassium lactate, giving a solid film on membrane II, and partly into glycogen. The solid film is, however, being destroyed by alkali, and the tension developed by this film is slowly decreasing with time. A rapid release at this stage destroys the tension. The surface energy may either be dissipated as heat or may be stored by way of the activation of lactic acid molecules, which pass into the precursor A according to the reaction: lactic acid \rightleftharpoons B \rightleftharpoons A. This reaction can proceed only so far as the equilibrium position.

On the other hand, an extension during this stage brings a larger quantity of lactic acid into contact with the anisotropic surface, and hence diminishes the number of activated lactic acid molecules which would normally pass into A. Thus an extension tends to drive the reaction to the right, while a shortening works in the opposite direction (equation 2, p. 46).

Thus the energy liberated will be high when the muscle is released during stimulus or extended after stimulus, and low when extended during stimulus or released after stimulus. These consequences are in general agreement with the experimental results of Fenn (22) and of Azuma (19).

Changes in the Tension as the Result of Movement of the Muscle during Stimulus.

The effect of a sudden release of a muscle fibre during isometric tetanus has already been shown to be in accord with the view that a solid film is produced. An extension from one length to another when the muscle is under isometric contraction produces changes in tension which are also in accord with this assumption. Gasser and Hill have shown that after a sudden stretch from one length to another the tension at first fell back to the initial value for the shorter length, and then slowly rose to the isometric tension for the greater length. If, however, the stretch was slow the tension at every moment was that corresponding to the equilibrium values of the tension-length diagram.

The behaviour of the solid film on stretching will depend on its tensile strength. As the area increases, the expansion may occur without rupture, or the film may break irregularly over certain segments. In the former case, it would be expected that the tension would be unimpaired on return to the longer length. On the other hand, if the film be disrupted it is

doubtful if the initial tension would be attained after the stretch. It thus appears that under the experimental conditions of Gasser and Hill the rupture of the film does not occur on stretching. The equilibrium value for the tension at the new length is imagined to be set up by a molecular rearrangement in the solid film and the liquid crystals. In the course of this there will be a further action between the lactic acid and the material composing the liquid crystals. With a very slow stretch, the rate of rearrangement is such that the equilibrium value for the tension is attained throughout. The maxima obtained with stretches of intermediate rapidity are best explained as due to elastic-viscous phenomena. The effect of a shortening, followed by a quick stretch, has already been referred to.

I am much indebted to Prof. A. V. Hill for experimental data bearing on the problem of muscle contraction, and for his kind advice on the physiological aspects of this paper.

Summary.

A mechanism of muscular contraction is proposed, in which it is suggested that the tension generated on applying a stimulus to a muscle fibre is due to the formation of a solid film on the surfaces of the ultimate fibrils of the muscle.

The evidence bearing on the occurrence of molecular orientation in muscle fibre is collected together, and it is concluded that liquid crystals composed of long-chain carbon compounds are present in the anisotropic segments of the muscle. The molecules of the long-chain carbon compounds are orientated with their chains in a direction parallel to the axis of the fibre.

The manner in which a solid film might be produced on the surface of the anisotropic segments by the action of lactic acid and the conditions under which a tension could be developed are discussed.

It is suggested that glycogen is converted into potassium lactate according to the series of reversible reactions, $A \rightleftharpoons B \rightleftharpoons \text{lactic acid}$, where A is a product formed from glycogen and B is an intermediate active form of both A and lactic acid. The direction of the chemical reaction may be influenced by alteration of the surface energy of the membranes during movements of the muscle. When the energy liberated during the conversion of glycogen into lactic acid is not utilised in doing external work, it may be stored, in part, by a reversal of the above reactions.

A simple model of an anisotropic segment of a muscle fibril is given, in which these conclusions are embodied. It is shown that the assumption of the production of a solid film, on the anisotropic segments, by the action of

lactic acid, is in accord with the experimental results on the force exerted, and the total energy liberated by a muscle when stimulated under various mechanical conditions.

REFERENCES.

- (1) H. S. Gasser and A. V. Hill, 'Roy. Soc. Proc.,' B, vol. 96, p. 398 (1924).
 - (2) A. V. Hill, 'Roy. Soc. Proc.,' B, vol. 98, p. 506 (1925).
 - (3) Langmuir, 'Journ. Amer. Chem. Soc.,' vol. 39, p. 1848 (1917); Adam, 'Roy. Soc. Proc.,' A, vol. 99, p. 336 (1921); A, vol. 101, pp. 452, 516 (1922); A, vol. 103, pp. 676, 687 (1923); A, vol. 106, p. 694 (1924).
 - (4) Biedermann, 'Electrophysiology,' p. 49 (1895).
 - (5) Engelmann, 'Pflüger's Arch.,' vol. 7, pp. 33, 155 (1873); vol. 11, p. 432 (1875).
 - (6) Hürthle, 'Pflüger's Arch.,' vol. 126, p. 1 (1909).
 - (7) Lehmann (1914). G. Friedel, *see* (11).
 - (8) Parnas, 'Biochem. Zeit.,' vol. 22, p. 411 (1909); vol. 56, p. 17 (1913).
 - (9) Rosenheim, 'Biochem. Journ.,' vol. 8, p. 121 (1914).
 - (10) Maclean, 'Lecithin and Allied Substances,' p. 124 (1918).
 - (11) G. Friedel, 'Ann. de Physique,' IX, vols. 17-18, p. 304 (1922); *see also* Lehmann, 'Flüssige Krystalle,' Leipzig (1904).
 - (12) E. Hückel, 'Physikal. Zeit.,' vol. 22, p. 561 (1921).
 - (13) E. Friedel, 'C.R.,' vol. 180, p. 269 (1925).
 - (14) De Broglie and E. Friedel, 'C.R.,' vol. 176, p. 738 (1923).
 - (15) Piper and Grindley, 'Proc. Phys. Soc.,' vol. 35, p. 269 (1922); *see also* McBain, 'Nature,' vol. 113, p. 534 (1924); vol. 114, p. 49 (1924).
 - (16) Müller and Shearer, 'Journ. Chem. Soc.,' vol. 123, pp. 2043, 3152, 3156 (1923); Gibbs, 'Journ. Chem. Soc.,' vol. 125, p. 2622 (1924); Garner and Randall, 'Journ. Chem. Soc.,' vol. 125, p. 881 (1924); Garner and Ryder, 'Journ. Chem. Soc.,' vol. 127, p. 720 (1925).
 - (17) Garner, 'Nature' (1925).
 - (18) Lillie, 'Amer. Journ. Physiol.,' vol. 41, p. 126 (1916).
 - (19) Azuma, 'Roy. Soc. Proc.,' B, vol. 96, p. 338 (1924).
 - (20) A. V. Hill, 'Physiol. Rev.,' vol. 2, p. 316 (1922); 'Journ. Physiol.,' vol. 45, p. 133 (1921).
 - (21) A. V. Hill, 'J. Physiol.,' vol. 60, p. 237 (1925).
 - (22) Fenn, 'J. Physiol.,' vol. 58, p. 175 (1923); *ibid.*, vol. 58, p. 373 (1924).
-

The Effects of Calcium and Potassium Ions on Urine Secretion, as Studied in the whole Animal.

By L. BRULL and F. EICHHOLTZ.

(Communicated by Prof. E. H. Starling, Foulerton Professor of the Royal Society.—Received August 6, 1925.)

(From the Physiology Institute, University College, London.)

In a previous paper Eichholtz and Starling (1) have described the effect of Ca and K ions on the kidney connected with the heart-lung preparation. They have shown that these salts, when acting together, have definite results on the volume and the composition of the urine yielded by the isolated gland.

These effects are :—

- (1) Increase of urine flow.
- (2) Increase of Cl percentage in the urine up to the chloride level of the serum.
- (3) Stoppage of the inorganic P excretion produced by the injection of inorganic P.

Attempts have been made to see whether and to what extent these statements can be confirmed on the whole animal. Thirty-five experiments have been performed.

METHODS.

Our experiments were performed on dogs, anæsthetized by morphia and chloralose. A little chloroform-ether was given until the chloralose was injected. The animals were on a variable diet before the experiments.

Blood pressure was measured from the carotid, urine flow by catheterizing both ureters separately. In all experiments the left kidney was denervated from the back. When the pituitary body was removed, the way of approach was the temporal one. The chemical determinations were performed according to the methods mentioned in Starling and Verney's paper (2).

EXPERIMENTAL RESULTS.

Two points are to be considered in this paper, viz. :—

- (1) Effect on water output. (2) Effect on chloride percentage.

Since in most cases these elements seem to behave independently, we shall deal with them singly. The question of the phosphate output in the whole

animal has been the object of a special study and will be treated of separately.

I. Water Output.

Under the experimental conditions mentioned above, the average urine flow in a dog of 10 kgr. is about 1 c.c. per 10 minutes per kidney. On the denervated side it is generally higher, and the spontaneous or artificially produced variations in volume are often more marked than in the intact gland.

The following figures give an idea of the average results of intravenous infusions of isotonic NaCl, KCl, and NaCl + CaCl₂ :—

Experiment I.—Dog, 7 kgr.

Time.	Arterial pressure.	Cubic centimetres urine right kidney.	Cl per cent. right kidney.	Serum.		
				Cl per cent.	Blood hæm. per cent.	Total solids per cent.
10-15				0-72	100	6-81 200 c.c. tap water <i>per os</i> .
12-15	130	0-6	0-11			4 c.c. isot. NaCl per minute intravenously.
12-35	135	1-3				
13-00	135	2-0	0-62			
13-20		2-5			86	
13-30	135	3-2	0-85			2 c.c. isot. NaCl.
13-50	140	6-7	0-89			
14-05	165	15-0	0-93	0-73	86	5-33 2 c.c. isot. KCl per minute.
14-15	130	19-5	0-91			2 c.c. isot. NaCl.
14-25	145	4-6	1-05			
14-35	145	2-5	1-21	0-80		4-875 2 c.c. isot. CaCl ₂ per minute.

Notice.—In all the experiments, the volumes of urine indicated are calculated per ten minutes and put down on the graphs and figures on the time (at which) the collection is completed.

These figures show that gradual infusion of large amounts of NaCl increase the water output, but not to a high extent. KCl has a much more pronounced effect, and this effect is not increased by Ca.

It was shown by Starling (2) and Eichholtz (1) (*loc. cit.*) that K may increase the water output from the isolated kidney, and by L. Brull (3) on the whole animal that Ca alone does not possess that property. When KCl and CaCl₂ are given together, it seems that the K ion plays the biggest rôle as regards urine volume. As to the mechanism of this action on water output, we are unable to supply a complete explanation.

The following figures of hæmoglobin and total solids of the serum, taken

during Na and K diuresis, show that the relatively greater effect of the latter is not due to hydræmia.

	Total solids per cent. in serum.	Hæmoglobin per cent.	Urine flow in 10'
Before any infusion	6.520	105	1.0
After isotonic NaCl	6.020	80	2.0
After isotonic CaCl ² + KCl.....	6.073	95	5.0

It is remarkable that in several experiments we found a divergence between the figures of total solids of the serum and the percentage of hæmoglobin. After CaK the serum solids remain on the lowered level produced by the constant infusion of fluid, but the hæmoglobin content often recovers partially or totally its previous level.

Barcroft and his collaborators (4) have suggested that the spleen may be considered as a reservoir of red corpuscles. They find that a constriction of this organ's vessels may throw into the circulation a significant amount of hæmoglobin. As it has been found by one of us (3) that the spleen is more sensitive than other organs to the vaso-constrictor effect of Ca, it might be that, when large amounts of CaK are infused, this effect appears first in the spleen and modifies the hæmoglobin percentage of the blood without affecting the composition of the serum.

II. Chloride Output.

A. *Intact Animal*.—In the L.H.K.* preparation Ca + K tend to change the urine into a glomerular filtrate, *i.e.*, the Cl percentage becomes the same in the urine and plasma.

The question of the output of Cl appears to be much more complicated than the study of the water output. In Experiment II everything looks quite simple.

The Cl percentage rose to about the serum level, together with the increased urine flow, and one would draw the conclusion that in the whole animal as well as in the isolated kidney Ca + K prevent tubular reabsorption of chlorides.

* *I.e.*, lung-heart-kidney preparation.

Experiment II.—Dog, 7.5 kgr.

Time.	Arterial pressure.	Temperature.	Cubic centimetres urine left kidney in 10'.	Cubic centimetres urine right kidney in 10'.	Cl per cent. urine left kidney.	Cl per cent. urine right kidney.
11.15	120	35	0.6	0.4	0.13	0.08
11.30	120		0.6	0.4		
11.36						100 mgr. Ca { isotonic 100 mgr. K { intravenously.
11.45	120		1.0	0.4	0.37	0.37
12.00	110-120	35.5	1.7	1.3	0.40	0.29
12.15	110		1.8	0.9	0.69	0.27
12.30	105	36	1.7	0.9	0.77	
12.45	100	37	1.3	0.8	0.79	0.48
13.05	130		1.5	0.4	0.13	} 0.08
14.05	130	38	0.5	0.5		

But in other experiments the results were just the opposite, as shown by the following figures :—

Time.	Pressure.	Temperature.	Cubic centimetre urine left kidney.	Cubic centimetre urine right kidney.	Cl per cent. right kidney.
10.05	145	35	3.2	1.8	0.56
11.10	145		3.8	2.5	0.42
					200 mgr. Ca, 400 mgr. K in 50 c.c. (chlorides)
10.15	145		4.1	3.2	0.43
10.25	150	35.5	4.6	4.7	0.32
10.35	145		3.4	3.2	0.32
10.45	145		2.8	2.1	0.31
10.55	145		1.8	2.2	
11.05	140		1.4	1.9	0.28
11.15	140	36			

These variable effects appeared in several experiments, and it looked as if we should not get any definite or constant result. The question arose, for what reasons the L.H.K. gives results which one cannot reproduce in the whole animal. There were at least three possible answers to that question :—

- (1) In the whole animal one or more organs may prevent the effect of $\text{Ca} + \text{K}$ on Cl output.

- (2) The bulk of tissues may absorb the greater part of the injected $\text{Ca} + \text{K}$ or of one of these two elements, and prevent them from acting on the kidney in a sufficient concentration.
- (3) The kidney of Starling and Verney's preparation may not be a normal one.

To resolve the first problem we tried the effect of removing systematically the different organs of the body, so as to determine which was the organ whose interaction with the kidney caused the latter to behave differently from the isolated kidney. For two reasons we chose as our first point of attack the pituitary body.

Starling and Verney have shown (*loc. cit.*) that the urine of the L.H.K. is like a polyuric one, with decreasing Cl percentage, and that injections of pituitrin are able to change this kind of diabetes insipidus into a normal state as regards Cl and water output. By that statement the attention of physiologists is once more called to the possibility of the existence of a hormonal action of the pituitary body.

On the other hand, Verney (oral communication of yet unpublished experiments) found, among other facts, that after removal of this gland from the whole animal, the kidney loses in the next few hours its ability to concentrate Cl.

B. Chloride Output after Removal of the Pituitary.—This gave us the opportunity of getting a step nearer to the state of the isolated kidney.

We carried out several experiments which confirmed Verney's statement. The chlorides drop more or less rapidly down to nothing after the removal of the pituitary. Immediately after the operation the kidney may still be able to put out Cl in a concentrated form, when this ion is present in high amounts, but it reacts as well in this manner to NaCl as to KCl or CaCl_2 . After one or two hours, large infusion of NaCl or the small doses of KCl and CaCl_2 we used generally (about 10 mgr. Ca and 20 mgr. K per kgr.) remain without effect. The water output is but little changed and may be slightly increased. The following experiment is an example :—

Experiment 7.—Dog, 11 kgr. Pituitary removed at 10.30 h., 400 c.c. tap water *per os*.

Time.	Pressure.	Temperature.	Cubic centimetres urine left kidney.	Cl per cent. left kidney.
11.35	110	35.5		
11.55	110		0.85	traces
12.15	110		0.85	0
12.35	109	35.5	0.60	0
12.55	110		0.80	0
				150 mgr. Ca, 300 mg. K in 36 c.c.
13.15	115		2.55	0
13.35	110	35.5	2.40	0
13.55	110	35.0	1.50	0

C. *Chloride Output after Skinning or Evisceration*.—We then tried the effect of removing other organs. One animal was skinned. After the operation the pressure was 80 mm. and a constant infusion of NaCl was employed in order to get a better urine flow. CaK had no appreciable influence on the Cl percentage, which remained low throughout the experiment.

Three other animals were eviscerated, the liver being left. In one case the result was negative, in the two others the chlorides were definitely increased. But NaCl produced some effect as well.

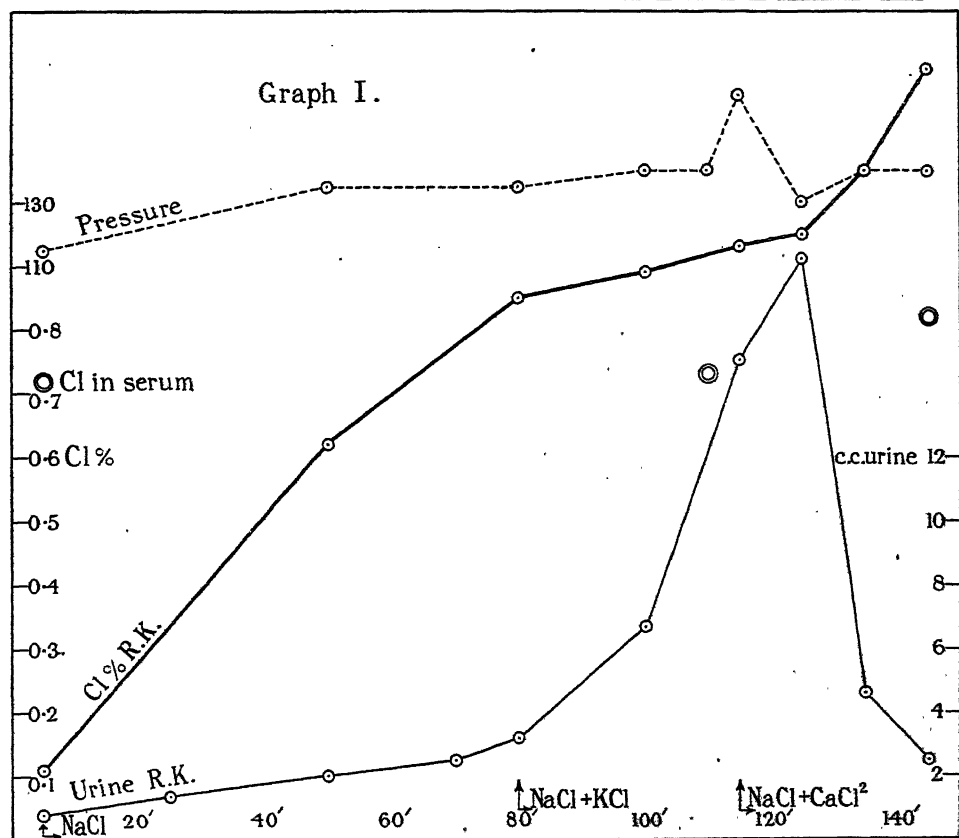
Nevertheless, although we had not the impression that evisceration had a specific effect on chloride percentage, the results were rather encouraging, since they suggested to us that it is easier to get reactions of the kidney to chlorides when a big bulk of tissues is removed.

D. *Chloride Output in the Intact Animal, after Big Doses of CaK*.—So doing, we were ready to put forward our second hypothesis: viz., that the amounts injected into the whole animal must be much higher than those used in perfusion experiments, owing to the fact that part of them may find its way into the tissues and be practically put out of action. But we met soon with a fundamental objection: the saturation of the tissues by K and CaCl_2 requires large amounts of salt, so that the body may become saturated with Cl, which in itself might be sufficient to account for the increased chloride output by the kidney. To avoid this difficulty the experiments were performed in the following way: a constant infusion of isotonic NaCl or Ca + K chlorides was carried out. If in those conditions NaCl could raise the Cl as well as the other two salts, the method was useless.

The following figures give the results on an intact animal:—

Experiment 29.—Dog, 9.5 kgr.

Time.	Pressure.	Cubic centimetres urine right kidney.	Cubic centimetres urine left kidney.	Cl per cent. left kidney.	Serum.		
					Cl per cent.	Hæmoglobin per cent.	Total solids per cent.
10.00					0.67		7.64 300 c.c. tap water <i>per os</i> .
11.50	120	0.5	1.0	0.67			
12.15	115	0.9	0.9	0.51			
12.40	115	0.8	1.0	0.47	0.67	105	6.52 5 c.c. isotonic NaCl per minute intravenously.
13.00		2.0	1.8				
13.25	120	1.8	1.9	0.87			
13.45		1.8	1.7	1.02	0.80	80	6.01
14.05	100-160	4.6	4.0	1.07		95	6.07 CaCl ₂ + KCl isotonic 2.5 c.c. of each per minute intravenously.
14.30	100-160	6.6	5.0	1.24			
14.50	120-190	2.6	4.25	1.36			
15.20	120-200	0.8	2.0	1.22	0.72	100	5.92 Infusions stopped.
15.55	150-170	0.5	1.3	1.15			
16.15	150-200	0.7	1.5	1.05			



Intact Animal. Constant isotonic infusions, 4 c.c. per minute. At beginning of experiment 200 c.c. tap water by the mouth.

The NaCl had but little effect on water output, but it increased the Cl percentage in the urine from 0.47 to 1.02. The further increase to 1.36 by CaK seemed to be the mere continuation of a regular curve. Other experiments gave the same results, as is shown in Graph No. I. (The big effect of CaK on water output should be noticed in this chart.)

E. *Chlorides after Removal of the Pituitary and Infusion of Large Amounts of CaK.*—As we had in our hands a means by which we could lower the Cl output—namely, the removal of the pituitary—we combined the constant infusion of the different salts with this operation.

In Experiment 19 a constant infusion of NaCl raised the chlorides to 0.95–0.83–0.94–0.95–0.98. The pituitary was then removed, and they dropped down to 0.49–0.42–0.31, the infusion being still maintained.

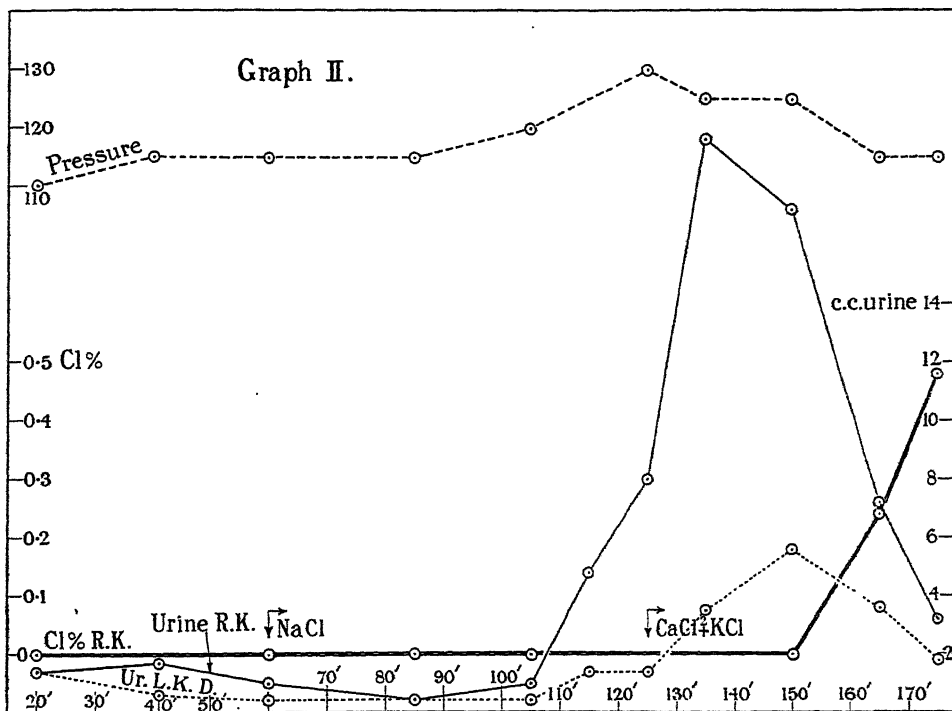
In other experiments the gland was removed from the beginning. The following is an example:—

Experiment 28.—Dog, 9 kgr.

Time.	Pressure.	Cubic centimetres urine left kidney.	Cubic centimetres urine right kidney.	Cl per cent. left kidney.	Serum.	
					Cl per cent.	Hæmoglobin per cent.
10.00						} 300 c.c. tap water <i>per os</i> . Pituitary removed.
11.00						
11.10	110	2.2	2.2	0.09	0.66	
11.30	115	3.5	1.2	0.09		
11.50	115	2.0	1.0	0.08		} 5 c.c. isotonic NaCl per minute intravenously.
12.15	115	2.0	1.3	0		
12.35	120	2.0	1.0	0.09		
12.45		4.6	0.5	0.06	0.71	
12.55	130	8.0	1.4	0.075		} CaCl ₂ } isotonic, 2.5 c.c. each KCl } every minute.
13.05	110–140	19.5	3.5			
13.20	110–140	26.0	8.7	0.075		
13.35	115	11.0	5.4	0.24		
13.45	115	3.2	1.9	0.48		

See also Graph No. II.

One can see that after 50 minutes CaK infusion, the chlorides rise from 0 to 0.48 per cent., whereas NaCl, infused during 125 minutes, was unable to produce any concentration. Controls were performed to find out whether one of these two ions plays a more important rôle than the other one.



Graph of the results obtained in Experiment 28. (V. Table.)

Experiment 32 is a K Control.—Dog, 11.250 kgr.

Time.	Press.	Cubic centimetres urine right kidney.	Cubic centimetres urine left kidney.	Serum.		
				Cl per cent. left kidney.	Cl per cent.	Hæmoglobin per cent.
11-20						100
12-30	110					300 c.c. tap water per os.
13-10	110		0.8			Pituitary removed.
13-40		0.4	0.8	0.12		5 c.c. isotonic NaCl per minute intravenously.
14-00		0.5	1.7	0.20		
14-15	110	0.5	2.2	0.21	0.68	
14-30		1.2	4.4			KCl } isotonic, 2.5 c.c. of each per minute.
14-45	110	1.4	6.2	0.25		
15-00	110	2.2	8.0		0.70	
15-10	100	2.3	12.3	0.26		again NaCl.
15-25	110	3.0	10.0			
15-40	110	3.8	10.6	0.14		
15-55	110	3.0	16.0	0.12	0.71	KCl } isotonic, 2.5 c.c. of each per minute.
16-15	100	2.8	17.7	0.08	0.69	
16-30	80-120	0.3	20.0			
16-40	80-120		9.5			CaCl ₂ }
16-50	80-120	0	6.0	0.40	0.70	

Even K + Na infusion during 75 minutes is unable to produce a definite rise in Cl percentage. On the contrary, the Cl, which rose in the beginning, drops down to 0.08 at the end; KCl does not prevent the effect of the removal of the pituitary on chlorides output. Ca + K is more active, raising Cl from 0.08 to 0.40 per cent. in 35 minutes. We conclude that KCl may have, as in the isolated kidney, a small effect on Cl output; but it is far from being a definite one, and is not to be compared with the Ca effect, as will be seen from the following experiment.

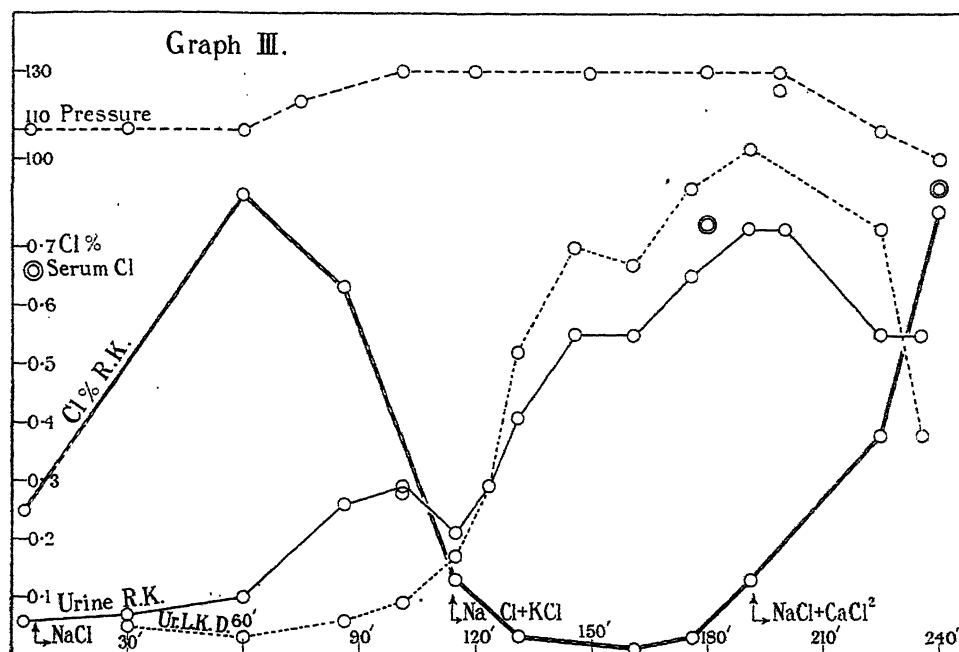
Experiment 34.—Dog, 7 kgr.

Time.	Press.	Urine right kidney.	Urine left kidney.	Cl per cent. right kidney.	Serum.			
					Cl per cent.	Hæmo- globin per cent.	Total solids per cent.	
10-15					0-66	100	200 c.c. tap water <i>per os.</i> Pituitary removed.	
11-05							NaCl isotonic, 5 c.c. per minute intra- venously.	
11-20								
11-45	110	0-9		0-25				
12-05	110	1-4	0-9					
12-35	110	2-0	0-6	0-79				
12-50	120	5-3	1-4				KCl } isotonic. NaCl } 2-5 c.c. of each per minute.	
13-00		4-3	1-1	0-63				
13-10	130	5-6	1-8					
13-20		5-8	2-3	0-28				
13-30	130	4-2	3-4	0-13	0-75	82-0		5-63
13-40	130	5-9	5-6				CaCl ₂ } isotonic. NaCl } 2-5 c.c. of each per minute.	
13-50	130	8-1	10-3	0-03				
14-00	130	11-0	14-0					
14-10	130	11-0	13-2	0-00				
14-25	130	13-0	16-0	0-03				
14-45	130	14-5	17-5	0-14	0-76	88-8	6-41	
14-55	80-180	14-5	19-5				CaCl ₂ } isotonic. NaCl } 2-5 c.c. of each per minute.	
15-20	110	11-0	14-4	0-48	0-80	86-2		6-29
15-25	80	11-0	7-6	0-76				

See also Graph No. III.

In the beginning the kidney seems still to possess part of its previous power of concentrating Cl, but it loses it gradually, and the chlorides drop down to 0.13 per cent. Na + K do not change the situation, even after 85 minutes, whereas Ca + Na increase the Cl level up to 0.76 per cent. in 30 minutes, the chlorides of the serum being 0.80 per cent.

A final question remains to be answered. In all the experiments in which



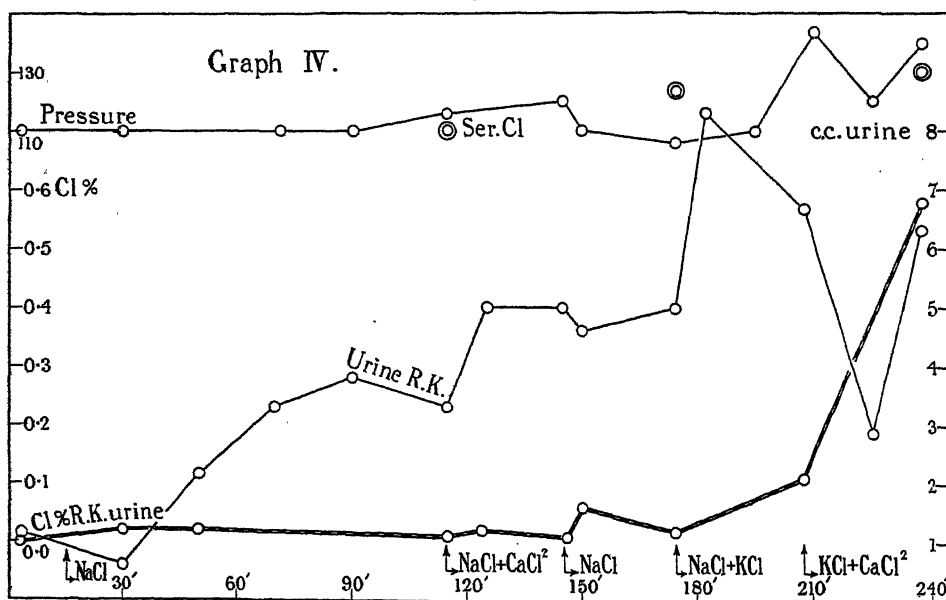
Graph of results obtained in Experiment 34. (See Table.) Pituitary removed. Constant infusion of isotonic chloride solutions, 5 c.c. per minute.

Ca had such striking effects, K was given previously. Starling and Eichholtz have called attention to the fact that the action of Ca on the isolated kidney sets in much more quickly when working on a background of K. Therefore it was possible that the good result obtained in Experiment 34 with CaCl_2 was due in part to the previous administration of KCl. The following figures show that Starling and Eichholtz's statement can be confirmed on the whole animal.

Experiment 35.—Dog, 8 kgr.

Time.	Arterial Pressure.	Cubic centimetres urine right kidney.	Cubic centimetres urine left kidney.	Cl per cent. right kidney.	Serum.		
					Cl per cent.	Hæmoglobin per cent.	Total solids per cent.
10-00						100	6.98
10-50	110	1.2		0			200 c.c. tap water <i>per os</i> . Pituitary removed.
11-05							NaCl isotonic, 5 c.c. per minute intravenously.
11-45	110	0.6	0.4				
12-05	110	2.1	0.9	0.02			
12-25	110	3.2	1.3				
12-45	110	3.8	2.1				
13-05	115	3.3	4.5	0.01	0.70	76.9	5.38
13-20	120	5.0	10.3	0.02			NaCl isotonic, 2.5 c.c.
13-40	110	5.0	9.1	0.01		80.6	5.16
13-50	110	4.6	11.2	0.06			CaCl ₂ isotonic, 2.5 c.c. per minute.
14-10	115	5.0	11.9	0.01	0.77	71.4	5.01
							again NaCl.
14-25	110	8.3	5.3				NaCl isotonic, KCl isotonic. 2.5 c.c. of each per minute.
14-47	120-170	6.7	4.1	0.11	0.77		
15-02	90-150	2.9	7.3				CaCl ₂ +KCl isotonic. 2.5 c.c. of each per minute.
15-12	100-180	5.8	2.3	0.53	0.80	71.4	4.77

See also Graph No. IV.



Graph of results obtained in Experiment 35. (See Table.)

These figures show that whereas in Experiment 34, 300 mgrs. Ca given in 30 minutes raised the chloride percentage of the urine from 0.11 to 0.76, in Experiment 35 the same amount remained without result. All conditions were similar, except that in the first case K had been given previously in considerable amounts. On the other hand, when Ca alone was inactive, CaK, given afterwards, raised the chlorides from 0.11 to 0.53 per cent. The view that Ca is only efficient when working on a background of K is therefore plainly supported by the experiment on the whole animal.

The last hypothesis put forward, "Is the kidney of the lung-heart-kidney preparation a nephritic organ giving abnormal reactions which are not reproduced by the whole animal?" may therefore be answered in the negative.

Summary and Conclusion.

Attempts have been made to see whether and in what conditions Starling and Eichholtz's results obtained on the lung-heart-kidney preparation with Ca + K could be confirmed on the whole animal.

It was found that *in the intact anaesthetised dog* :—

(1) KCl injected intravenously increases the urine flow to a much higher extent than injection of corresponding amounts of NaCl. This increase is not due to hydræmia, nor to changes in blood pressure.

(2) KCl or CaCl_2 , either isolated or combined, have no constant nor specific effect on chlorides output. The intact animal behaves towards them as it does towards NaCl, using its general power of concentrating chlorides.

When the pituitary body is removed, and the kidney has consequently lost its power of concentrating chlorides (Verney), it reacts to CaK in the same way as was found by Starling and Eichholtz in the lung-heart-kidney preparation, that is to say :—

- (a) Ca + K, or even K alone, increase the urine flow, whereas Ca alone does not, and NaCl has a much smaller effect.
- (b) Ca + K, or, more definitely, Ca working on a background of K, increases the chlorides percentage up to the chloride level of the serum.

Since the whole animal, after removal of the pituitary body, reacts to Ca + K in the same way as the isolated kidney of Starling and Verney's L.H.K. preparation, we conclude that by means of this preparation important reactions of the kidney can be studied and elucidated under simplified but still normal conditions.

We are much indebted to Prof. Starling for his help and advice throughout the experiments.

REFERENCES.

- (1) F. Eichholtz and E. H. Starling, 'Roy. Soc. Proc.,' vol. 98, p. 93 (1925).
 - (2) E. H. Starling and E. B. Verney, *ibid.*, vol. 97, p. 321 (1925).
 - (3) L. Brull, 'Arch. Int. M  dec. Exp  rim.,' vol. 1, p. 613 (1925).
 - (4) J. Barcroft, C. D. Murray, D. Orahovats, J. Sands and R. Weiss, 'Journ. of Physiol.,' vol. 60, p. 79 (1925).
-

*The Secretion of Inorganic Phosphate by the Kidney.—II.
Influence of the Pituitary Gland and of the Wall of the
Third Ventricle.*

By L. BRULL and F. EICHHOLTZ.

(Communicated by E. H. Starling, F.R.S., Foulerton Professor of the Royal Society.—Received August 6, 1925.)

(From the Physiology Department, University College.)

In previous experiments on the diuretic properties of calcium and potassium ions (1), we tried the effect of removing various organs from the whole animal, in order to investigate whether they exert some influence on the secretory functions of the kidney. We were struck by the fact that after removal of the pituitary body, the kidney may lose in the following few hours its power of secreting inorganic phosphorus. Since in those experiments inorganic salts, which may have an influence on P secretion, were injected, we started a separate study of the action of the pituitary on phosphate excretion.

METHODS.

Dogs only were used. They were an  sthetised with 0.01 gr. morphia and 0.1 chloralose per kilogram, small amounts of chloroform-ether being given to start with. Blood pressure was measured from the carotid; blood samples taken from the femoral artery. All necessary infusions were made through the jugular vein. Urine was collected from both ureters and analysed separately, one kidney being denervated from the back (left side).

The determination of inorganic P in urine and serum was performed according to Briggs' modification of Bell-Doisy's method (2). Chlorides were estimated by Millard Smith's method (3). H  moglobin, total solids, chlorides and urea

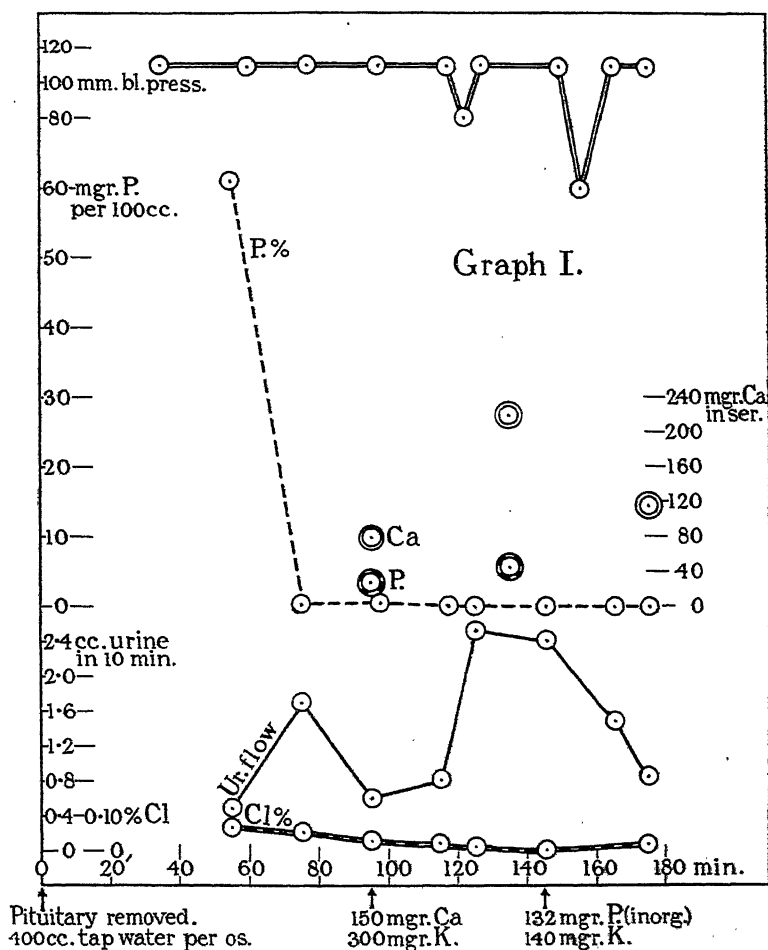
determinations were performed several times during the experiments. The urea was estimated by the urease method.

The figures of Cl are given in NaCl per cent., phosphates in mgr. P per 100 c.c. In all tables and charts the indicated figures of urine flow and composition given at a certain time correspond with the urine collected up to that time.

EXPERIMENTAL RESULTS.

The effects of removal of the pituitary body on the secretion of inorganic phosphates will be better understood if we quote here the experiment in which our attention was first drawn on the matter.

A dog of 11 kgr. prepared as described above, received 400 c.c. tap water *per os*



Graph of Experiment 7.

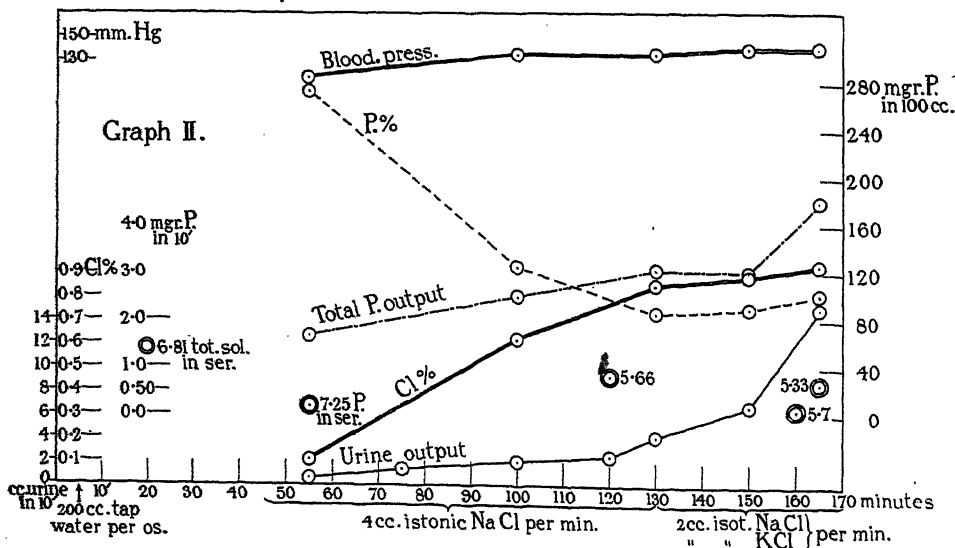
as soon as it was anæsthetised. The pituitary body was then exposed, after separation of the left temporal muscle from its insertion, cutting of the zygoma, removal of the bone over a large area in the temporal region, excision of the exposed dura mater and lifting of the brain. The gland was then seized by its pedicle and removed. The results are given in Graph I.

It will be seen that after the urinary tract had been washed out by 2 or 3 c.c., the secreted urine no longer contained inorganic P, so that the kidney not only had lost its power of concentrating phosphorus, but there was no simple filtration of the inorganic P from the blood, or, if some passed the glomeruli, it was reabsorbed lower down in the tubules.

The possibility that a gland, which has the reputation of influencing the growth of bones, might have such a striking effect on the elimination of the P induced us to repeat the experiment. Moreover another reason stimulated our research, namely the possible explanation of the fact that the isolated kidney of Starling and Verney's heart-lung-kidney preparation is unable to put out inorganic P, whereas in several other respects it behaves physiologically. More than 30 experiments were performed, and the pituitary body was removed in 28 cases. As in 16 cases of removal positive results were obtained, it may be worth while discussing the figures.

Significance of the Experimental Conditions.

A number of factors might possibly account for the extinction of the P secretion, namely: the narcosis, the previous administration of water *per os*,

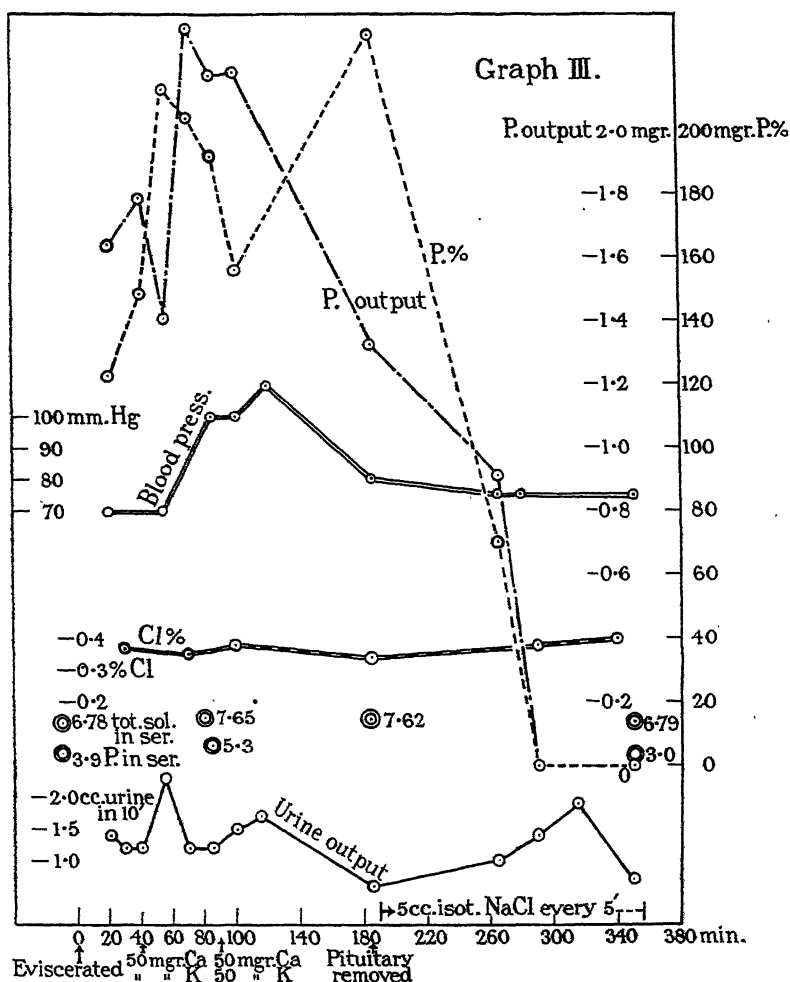


Graph of Experiment 36.

the intravenous infusion of NaCl, the operation—in short, every one of the experimental conditions under which we worked.

It would be too long to give here all the details of the numerous controls which were performed. The main results are summarized in Graphs Nos. II, III and IV.

It is evident from the figures in Graph II that neither the anaesthesia, nor the administration of water *per os*, nor the intravenous infusion of large amounts of isotonic NaCl, diminishes the output of P, even in a long-lasting experiment. Moreover, the chloralose or the chloroform-ether given in the beginning are not responsible for the observed phenomenon, since animals anaesthetized by



Graph of Experiment 16.

somniphene may exhibit it as well. On the other hand, the abolition of the secretion does not occur from the moment the animal is under the anæsthetic, but occurs only after the removal of the pituitary.

The results in Graph III show that the phosphates excretion may still be normal after an experiment lasting three hours, but is at once abolished by extirpation of the pituitary.

Influence of Operation and Shock.

The removal of the pituitary is not an operation without import, as is shown by the low blood pressure (often 80–90 mms. of Hg) which followed it, especially in our earlier experiments when we were still unfamiliar with the technique of the operation.

Graph III shows that such a severe operation as evisceration may lower the blood pressure to the same extent (70 mms.) without affecting the P secretion, but that this secretion is stopped at once by subsequent removal of the pituitary.

In several of these experiments the effect of removal of the pituitary was negative, in spite of the fact that the way of approach to the gland was exactly the same as in all the others, showing that the surgical interference could not account in itself for the change in the kidney's activity. We shall come back below to the discussion of those negative results. The operation may often be performed without affecting the blood pressure, but the effect is still the same.

The fact that, even after varying or eliminating all the experimental conditions under which we have worked, the phenomenon we are interested in still appears, leads us to conclude that "*the operation terminated by the removal of the pituitary body is responsible for the fall of the inorganic P percentage in the urine.*"

But that operation is in itself a significant interference in the normal state of the animal. The trephining, and especially the opening of the arachnoid spaces by removal of the dura mater and the consequent draining of the cerebrospinal fluid, may produce changes whose importance is but little elucidated. On the other hand, the lifting of the brain and its eventual damage must be taken into account.

For several years past many authors, after Camus and Roussy (4), have drawn attention to the possibility of reproducing some of the so-called effects of removal of the pituitary body by mere injury to the base of the brain, and more particularly to the nervous structures of the tuber cinereum.

It was therefore necessary to determine the influence of these factors by special experiment.

Influence of Trephining, Draining of the Cerebro-spinal Fluid, and Lifting of the Brain.

In order to ascertain to what extent the special conditions created by a large removal of the dura mater—namely, the draining of the cerebro-spinal fluid, the change of pressure at the surface of the brain, with all their eventual consequences—might account for the extinction of the P secretion, the pituitary gland was exposed for about two hours in six experiments before it was removed, or before puncture of the third ventricle was carried out.

In no one of those controls was the P output decreased by the exposure. In four of them it was abolished subsequently by the extirpation of the gland, in one the removal had no effect, and in the last one the P secretion disappeared after puncture of the tuber cinereum. The results of one experiment are given in Graph IV.

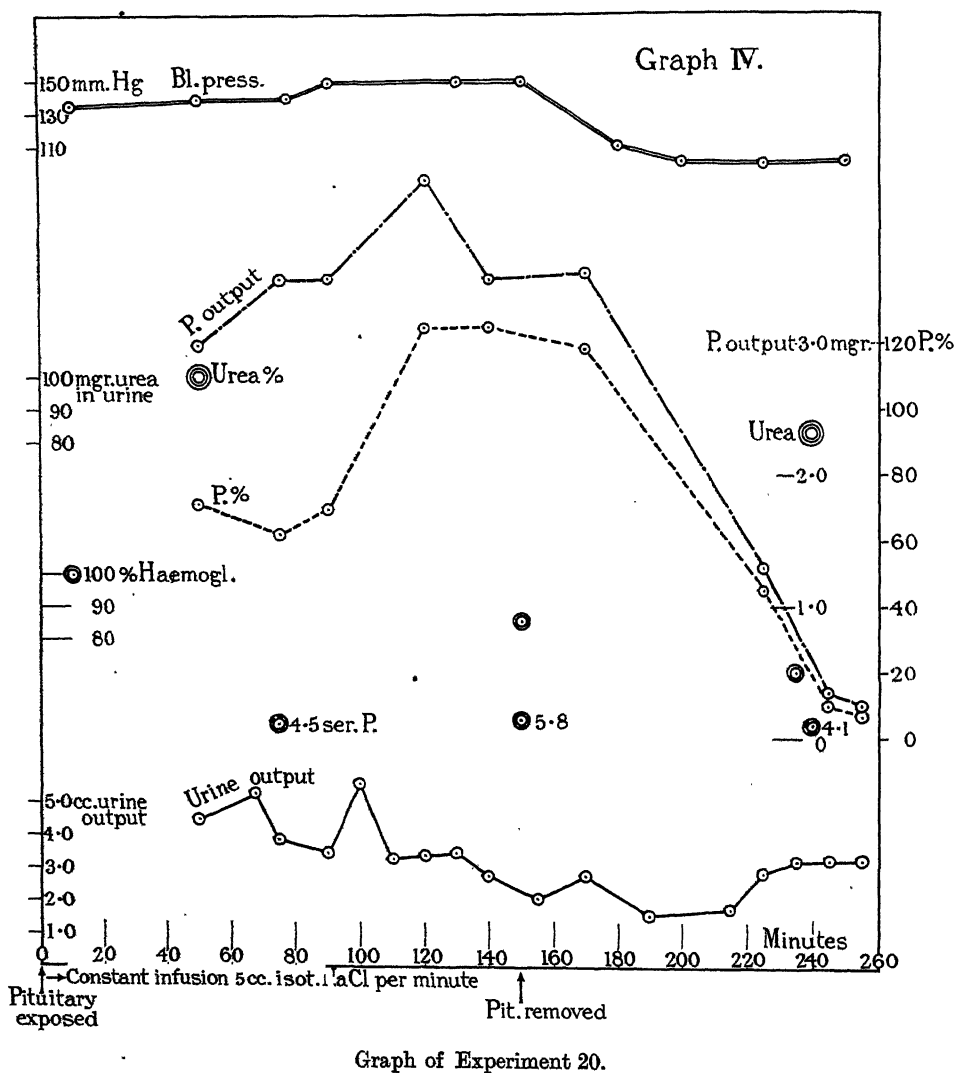
It will be noticed that the P output in this experiment is unchanged after two hours' exposure, the percentage being increased from 65 to 120, when both suddenly drop down after the removal of the pituitary. The lowering of blood pressure occurring in this experiment does not appear in other cases which gave similar results.

In another experiment the cerebro-spinal fluid was drained by introduction of a hollow needle into the fourth ventricle. After a sudden outflow the fluid went on dropping regularly during two hours, giving altogether 16 c.c. The P output, which was maintained at the figures of 0.30—0.25 (mgr. P in 10 minutes per kidney) during the draining, immediately fell to 0.03—0.01—0.00 after puncture of the tuber cinereum. Furthermore, as has been already pointed out, we obtained eight negative results of pituitary removal, although in all those experiments the animals were observed during three and more hours after the extirpation.

If these experiments are not sufficient to exclude the possibility of a hormone, influencing the kidney activity, being secreted into the circulation *via* the cerebro-spinal fluid, they seem to afford sufficient evidence that the draining off of the fluid is not the cause of the sudden extinction of P secretion, which may be produced by removal of the pituitary.

Harm done to the brain by lifting can scarcely be invoked, since in our control experiments the temporal lobe was kept elevated from the beginning by a plug of cotton-wool without any influence on P secretion. The trephining was always large enough to allow lifting of the brain without compressing it against the skull. The *post mortem* did show in several cases some blood effusion in sections of the brain, but they became more and more negligible as our

technique improved. On the other hand, in one experiment we intentionally injured the temporal lobe by pushing the cautery into its cortex in six different



places in the region where the lesions produced by the operation were generally found. This manipulation had no effect on P output.

We have deemed it advisable to deal at some length with these experimental data in view of the fact that the phenomenon we were investigating had not previously been observed, and we were anxious to assure ourselves that it

was not due to accidents of manipulation, apart from the injury purposely inflicted on the pituitary body.

Is the Fall of the Phosphate Output due to the Extirpation of the Pituitary Body ?

After wide removal of the skull in the temporal region and lifting of the brain, the gland is very easily exposed. As it is difficult to stop bleeding in that region without causing local compression and damage to the brain, we avoided the use of any sharp instrument, and seized the gland and cut the pedicle by means of a long forceps. In some cases it was easy to remove the whole gland with the forceps. Since, however, the gland is slightly adherent to the *sella turcica*, it often comes apart or drops from the forceps, and the removal may be carried out only piecemeal. It was necessary, therefore, to control the extent of removal by subsequent microscopic examination of the base of the brain and of the dura mater in the *sella turcica*.

As already stated, in eight cases out of 24 the extirpation was not followed by the abolition of P secretion. Since we were unable to supply any explanation for those negative cases, we hoped that the histological control might give the solution of the problem.

In nine experiments complete series of histological slides of the base of the brain at the level of the stalk of the pituitary were made. The dura mater in the *sella turcica* was removed and sectioned. The removed gland was also cut to ascertain whether it included all the parts of the pituitary.

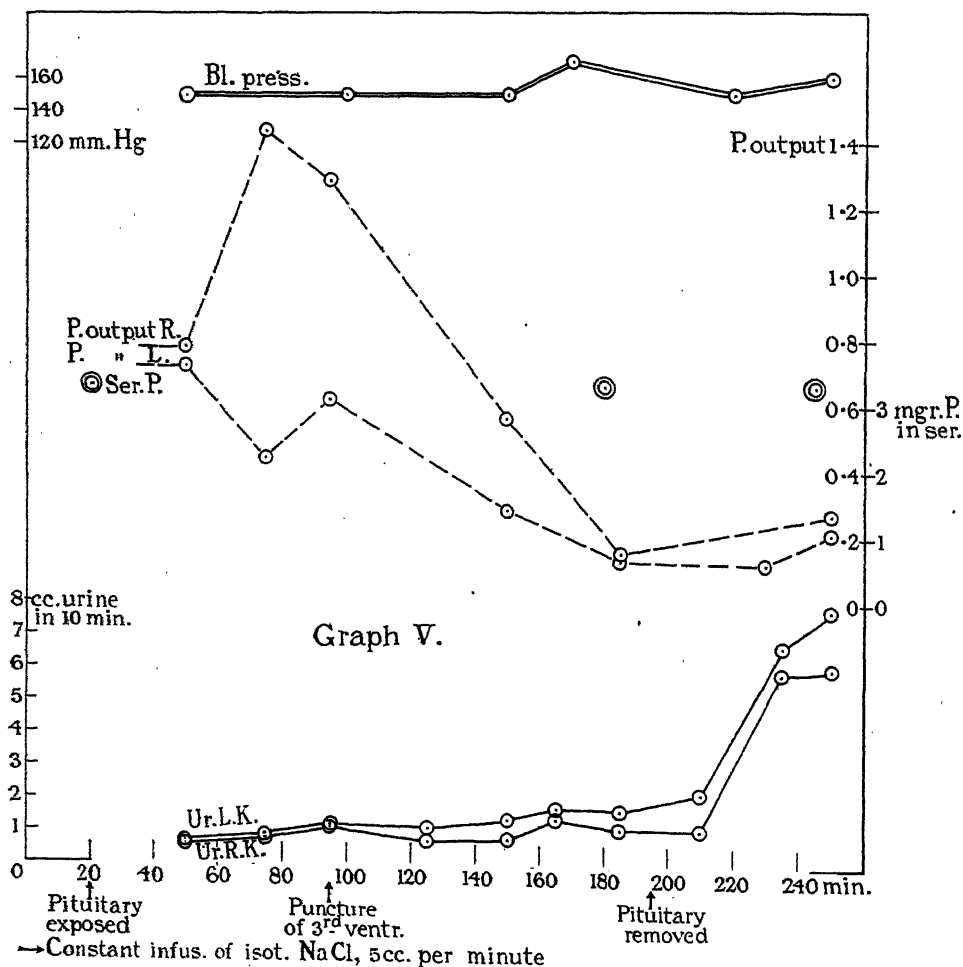
In eight cases practically nothing of the anterior lobe was found to be left. Small remains of the anterior lobe were generally found in the remainder of the pedicle, and sometimes in the *sella turcica*. But as a rule they did not correspond to more than about 5 per cent. of the total gland, and generally to less. In one case only was a more considerable part of the anterior lobe found in the sella (experiment 42). It should be noted that in that experiment the P excretion dropped. Remains of the posterior lobe so far as they could be recognised were never found.

On the whole the pituitary body was practically removed. The small fragments were very likely deprived of their normal blood supply, being surrounded and isolated by extravasated blood. Were it not for the 33 per cent. of experiments which were negative, it might provisionally be admitted that the observed change in the kidney secretion was the consequence of the removal. But even without considering the possible interference with the third ventricle, the negative cases had to be explained, and the histological control was far from clearing up the situation. There was no difference in the anatomical condi-

tions found after removal between the positive and negative cases. The less complete removal corresponded to one of the best positive experiments. As a consequence we were induced to direct our investigations elsewhere.

Influence of the Puncture of the Tuber Cinereum.

The first part of the operation was the same. After exposure of the base of the brain and sufficient delay to ascertain whether the P secretion remained more or less constant, a cautery of a diameter of $\frac{1}{2}$ mm. was pushed into the tuber cinereum, as nearly as possible at the level of the infundibulum. This operation was performed without touching the pituitary.



Graph of Experiment 46.

Anatomical control shows that the lesions varied from a narrow puncture surrounded by slight hæmorrhage and passing through the recessus infundibuli to a larger destruction of about 5 mm. diameter, damaging both the infundibulum and the tuber cinereum. A detailed study in order to ascertain which groups of nervous cells were destroyed was not performed. In three out of four experiments we found that a *lesion of the tuber cinereum, without any apparent harm to the pituitary body, abolished the inorganic P secretion by the kidney.*

The figures in Graph V show that whereas the previous P output was constant in the denervated kidney and increasing in the innervated one after 100 minutes' exposure, the puncture of the third ventricle suddenly brought this output down to a very low level without affecting the urinary flow.

Are we to conclude that, if a mere puncture of the tuber is sufficient to abolish P secretion, when we get the same result by extirpation of the pituitary, it is due to the fact that we cause injury or excitation of the base of the brain?

Or shall we admit that by puncturing the tuber we disturb the functions of the pituitary gland, for instance, by stopping the way of excretion of its products? We have no sufficient data to solve this problem. Nevertheless some more light may be thrown on the question by studying what happens when the pituitary is removed after puncture.

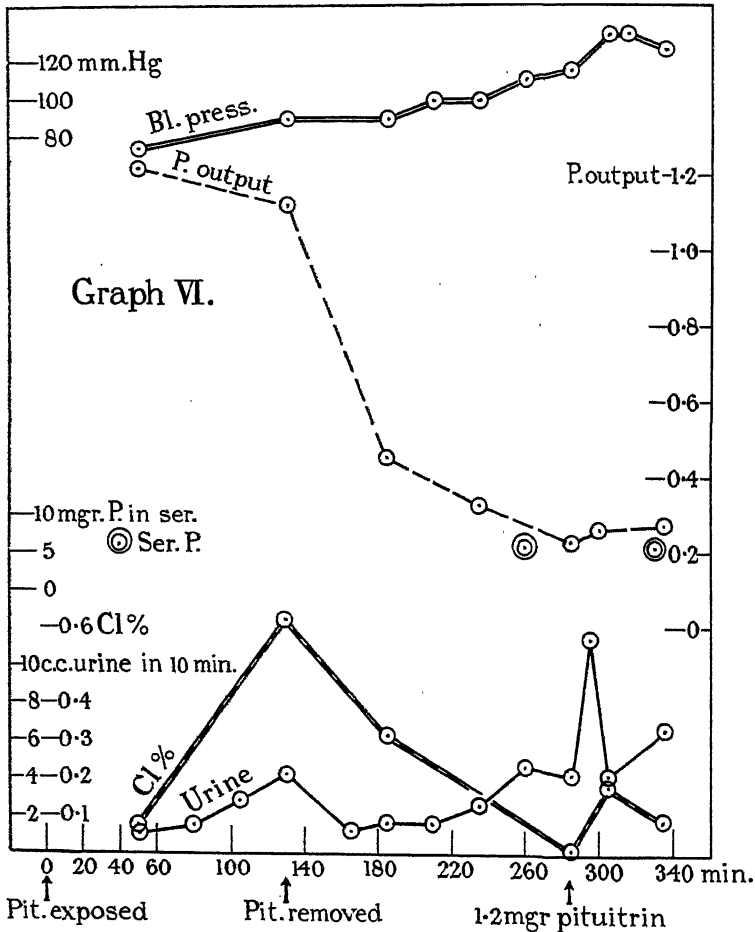
The Dissociation of the Effects on P Output on the one hand and on Cl Percentage and Water Output on the other.

Verney has found (oral communication of yet unpublished experiments) that in an anæsthetised dog extirpation of the pituitary body decreases the Cl percentage in the urine, and may produce polyuria within the next hour. These statements we have often confirmed, but we found that in many cases there was no parallelism between the behaviour of the phosphates and of the chloride. In some cases the extirpation lowered the Cl and P together.

In Experiment 43 the Cl percentage is raised by infusion of NaCl, but suddenly lowered by removal of the pituitary. The P output follows the Cl percentage in its fall. But in other cases the removal of the pituitary had no effect on chloride or water secretion, whereas it put an end to the P secretion.

Graph VII shows a steep fall of P percentage together with a large increase of Cl percentage due to NaCl infusion, and no important change in urine flow. The explanation of such a case might be that the small remainder of the gland which is left is sufficient to account for the Cl concentration,

the P elimination being presided over by some other mechanism. Camus, Roussy and others would deny that the pituitary has anything to do with polyuria. The most complete dissociation is shown in cases, such as that shown in Graph VIII, in which the puncture of the ventricle reduces the phosphates output to nothing, while the Cl is increasing owing to NaCl



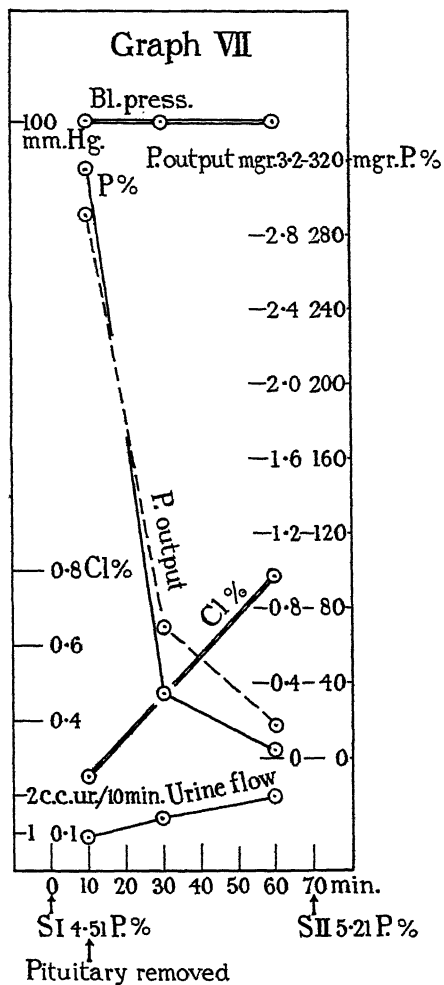
Graph of Experiment 43.

infusion. The Cl percentage is lowered to zero afterwards by removal of the pituitary.

We conclude, from Graph VIII, that the excretion of phosphate can be affected and abolished quite apart from any changes caused at the same time in the amount of urine or the concentration of chloride therein. It would,

however, be premature to conclude that we have to deal with two independent mechanisms.

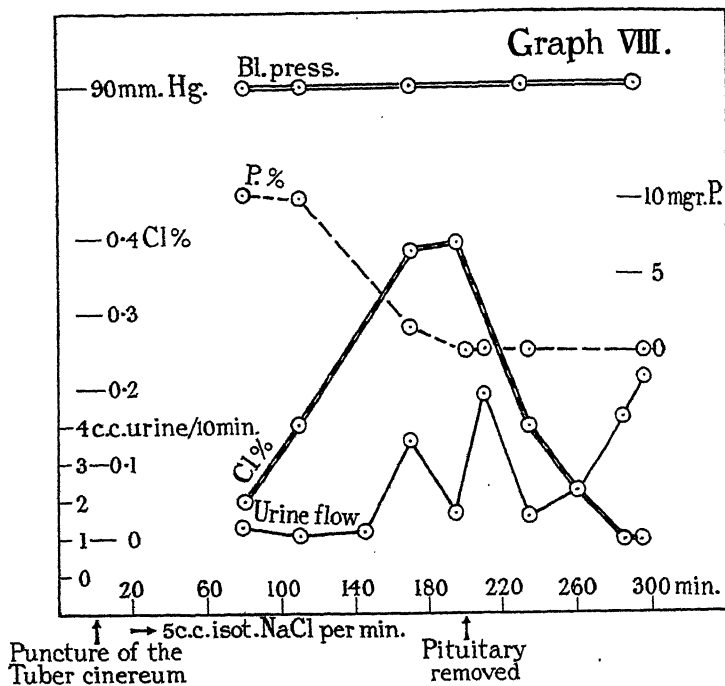
In all cases in which the two phenomena were dissociated, the phosphate secretion was affected first. On the other hand, as will be shown later, the



injection of pituitrin, which restores a high Cl percentage, has generally no effect on P output. In two cases, however, it did improve the P output, and these were cases in which larger amounts of pituitrin could be injected without causing an important drop in the total amount of urine secreted.

It seems, therefore, possible that if both Cl and P secretion depend on a

hormonal effect of the pituitary or of the tuber cinereum, the phosphates disappear sooner because they require a higher concentration of hormone than



Graph of Experiment 44.

the chlorides. The injection of pituitary extract would bring back the chlorides first for the same reason.

Negative results of puncture of the tuber cinereum are not very significant, so long as we are unable to localise the centre or glandular formation which influences the phosphate secretion.

Mechanism by which the Pituitary or the Wall of the Third Ventricle may Influence the Secretion of Inorganic Phosphate.

In the first place the control of the phosphate secretion might be a nervous mechanism. It could not be by direct nervous influence on the secretory cells of the kidney, since denervation of the kidney did not affect sensibly the results of the operative interference, which were manifest both in the normal and the denervated organ (Graph V). We cannot, however, exclude the possibility of an indirect nervous effect. The direct result of the operation might be on some unknown organ, which, in its turn, by some chemical means,

might influence the kidney functions. On the other hand, the effect of our operation may be to upset some hormonal mechanism.

The only direct method of detecting the secretion of a hormone is to get evidence of its presence in the venous blood coming from the organ which may produce it. An indirect method is to test whether extracts of the organ may reproduce its physiological effects when injected. We did not prepare extracts of the wall of the third ventricle, but we tested the effect of extracts of the pituitary body.

The following table (Experiment 22) shows the effect of injection of anterior lobe extract and afterwards of pituitrin. The anterior lobe injected corresponded to the extract of 25 ctgr. dry anterior lobe (Armour) prepared by the same manner as pituitary extracts are prepared, but without precipitation of the proteins. The pituitrin injected was 1 ctgr. posterior lobe extract (B.D.H.).

Table I.—Experiment 22. Dog. 9 kgr.

Time.	Blood Pressure.	Urine cubic centimetre/10 minutes, left kidney.	Inorg. P per cent.	Inorg. P mgr./10 min.
10.50	—	starts	—	—
11.17	—	2.7	—	—
11.30	—	—	—	Pituitary exposed.
11.40	120	1.0	293	2.93
11.55	105	1.4	—	—
12.00	—	—	5.0	Serum I.
12.30	—	—	—	Pituitary removed.
12.40	100	0.65	277	1.79
13.20	105	0.50	—	—
13.50	100	0.70	293	2.05
14.45	100	0.60	—	—
15.20	120	1.3	143	1.63 Anterior lobe extract.
15.45	—	1.3	—	—
15.55	—	—	5.2	Serum II.
16.00	130	1.4	57	0.86
16.50	—	0.7	71	0.50
				1 centigram pituitrin.
17.08	135	1.1	—	—
17.18	—	2.0	131	2.62
			5.8	Serum III.

In this experiment the phosphate secretion is not abolished after removal of the pituitary, but at the end of the experiment it is rapidly decreasing. The anterior lobe extract does not stop the decrease, but the pituitrin brings the phosphate output back to its previous level.

The effect of pituitrin looks very striking in this experiment. We were able to reproduce it in a similar case, but in both cases the phosphate secretion was not totally stopped previously. In many other experiments in which the phosphate excretion was totally or nearly abolished pituitrin had no effect, whereas it brought about a considerable increase of Cl concentration (*see above*, Graph VI, Experiment 43). The chlorides, which dropped to nothing after removal of the pituitary, rose after 20 minutes to 0.18 per cent., while the P remained on its low level. In one case, however, in which the P secretion was abolished by puncture of the tuber cinereum, and the Cl by removal of the pituitary, 5 mgr. pituitrin brought the Cl percentage back to its previous level, increasing the P percentage from 0 to 5 mgr. (Table II.)

Table II.—Experiment 48.

Time.	Blood Pressure.	Urine, cubic centimetres/10 minutes. Denerv. kidney.	Cl per cent.	Inorg. P per cent.	Inorg. P mgr. 10 min.
13.15	110	0.50	0.06	60	0.30 Serum I.
13.45	—	0.75	0.05	33	0.25 Serum II.,
14.10	105	—	—	—	Puncture of tuber cinereum.
14.35	110	0.50	0.35	5	0.03 Serum III.
15.05	110	0.60	0.72	3	0.018
15.35	110	0.60	0.97	—	—
15.40	—	—	—	—	Pituitary removed.
16.00	—	0.80	—	—	—
16.20	—	4.60	0.12	0	0 Serum IV.
16.30	—	11.30	0.08	0	0 } 1 ctgr.
16.40	—	3.60	0.15	5	0.04 } pituitrin.
16.50	—	3.90	1.01	5	0.18 Serum V.
17.00	—	5.20	0.96	5	0.25

The figures of phosphates in the serum were : 5.5 — 3.5 — 3.75 — 4.25 — 5.50. The concentration of 5 mgr. P obtained in the urine after the injection of pituitrin does not even reach the serum level, but owing to a large urine flow the total P output was increased to about the same amount as before the puncture of the third ventricle. The chlorides came back to the same concentration as before the removal of the pituitary.

It appears, then, that pituitrin may produce an increase of phosphate output, but this increase is not constant. In our experiments the P concentration of the urine never reached its previous level, by injection of pituitrin,

provided that the secretion was really abolished previously, whereas that of Cl often did.

The vasomotor property of pituitrin, which, in apparently the same conditions, was very variable from one case to another, often prevents the use of sufficient amounts of that substance. When too large amounts (say, 5-10 mgr. in a dog of 10 kgr.) of this extract are injected, the urine flow is lowered to such an extent that it is difficult to draw conclusions from the results.

These few positive results are insufficient to decide whether pituitrin may really make good the deficiency created in the kidney as regards phosphate secretion. Neither is the fact that chloride may reappear without phosphate a proof against a possible effect of pituitrin on P secretion. As was pointed out above, it might be a mere question of quantity, the secretion of chlorides requiring less pituitrin than that of the phosphates.

Changes in the Serum after Removal of the Pituitary or Puncture of the Tuber Cinereum.

In all experiments several determinations of inorganic P in the serum were carried out. The following figures show that the abolition of phosphate secretion is not due to a change of the inorganic phosphate level in the serum.

Table III.—Experiment 35.

Time.	Serum.			Urine innervated kidney.		
	Inorg. P.	Hæmo- globin, per cent.	Total solids, per cent.	Flow cubic centi- metres /10 min.	Inorg. P per cent.	Inorg. P mgr. 10/min.
10.15	4.51	100	—	0.9	295	2.70
11.05	Pituitary removed					
13.25	5.21	82	5.63	5.8	2.25	0.13
14.40	5.00	88.8	6.41	13.0	0	0
15.22	5.00	86.2	6.29	11.0	0	0

Constant infu-
sion of 5 c.c.
isotonic NaCl
per minute.

In a few experiments larger variations of inorganic phosphate in the serum appeared, but these changes were not of a constant type, increases occurring as well as decreases, so that they could hardly be taken into account.

As it is suggested by Eichholtz, Robison and Brull (5) that the secretion of inorganic phosphate may depend, at least in part, on the organic phosphates of

the serum, these phosphates were estimated in two experiments together with the inorganic.

Table IV.—Experiment 40.

Time	Serum.				Urine innervated kidney.			
	Inorg. P per cent.	Org. P per cent.	Hæmoglobin, per cent.	Total solids, per cent.	Flow cubic centimetres /10 min.	Inorg. P per cent.	Inorg. P mgr. 10 min.	Urea mgr. /100 c.c.
10.40	6.66	0.24	100	7.79	0.70	72.5	0.50	—
13.30	6.40	0.20	81	6.32	4.60	19.1	0.87	215.6
14.00	Pituitary removed.							
15.45	5.65	0.23	82	5.60	2.20	2.8	0.07	—
16.40	6.13	0.27	80	—	3.30	2.6	0.08	444.0

The figures of organic P are quite constant even after dilution of the blood by large isotonic NaCl infusion, suggesting the existence of a constant equilibrium between the phosphates of corpuscles and serum.

It is clear that the inability of the kidney to secrete phosphate in the second part of those two experiments does not depend on a change in the available percentage of either inorganic or organic P in the plasma.

Changes in the Secretory Powers of the Kidney.

It was shown above that after the kidney has lost its faculty of secreting phosphate, it may still be able to concentrate chloride, when this is provided by injection (Graph VII, experiment 34 ; Graph VIII, experiment 44 ; and Table I, experiment 22). The function of urea secretion seems also to be retained, as is seen from Table IV, experiment 40. It appears therefore that the kidney is not deficient in its other secretory functions after the P secretion is abolished.

Is it *unable* to secrete phosphates after removal of the pituitary or puncture of the third ventricle ? It is shown by Eichholtz, Robison and Brull (*loc. cit.*) that when organic phosphates are injected intravenously, after experimental abolition of inorganic phosphate secretion, by removal of the pituitary or injury of the tuber cinereum, those phosphates are put out in the urine in a concentrated form, and may be hydrolysed to a high extent by a phosphatase contained in the kidney cell, inorganic phosphate being secreted.

We have also tried the effect of injection of sodium phosphate after elimination of the normal P secretion. (Table V.)

Table V.—Experiment 44. Dog, 9·5 Kgr.

Time.	Blood Pressure.	Urine, innervated kidney				Serum/ Inorg. P	—
		Flow cubic centimetre /10 min.	Cl per cent.	Inorg. P per cent.	Inorg. P mgr. 10 min.		
11.20	—	—	—	—	—	—	Constant infusion 5 c.c. isol. NaCl per min.
11.40	90	1·3	0·05	10·5	0·13	—	Puncture of the tuber cinereum.
13.35	90	1·6	0·39	0	0	3·2	Pituitary removed.
14.40	90	2·3	0·15	0	0	2·8	
15.05	110	5·2	0·00	0	0	—	30 mgr. P (sodium phosphate).
15.15	—	6·2	—	—	—	4·3	} 100 mgr. P (<i>idem</i>).
15.30	100	6·5	—	2·5	0·18	—	
15.50	100	6·6	—	0	0	5·0	} 150 mgr. P (<i>idem</i>).
16.10	—	7·1	—	2·7	0·19	—	
16.20	100	8·5	—	5·2	0·44	10·2	
16.30	—	9·3	—	—	—	—	
17.00	95	8·1	0	22·5	1·80	—	
17.10	100	9·5	0	20·0	1·90	—	
12.35	—	—	—	—	—	4·2	

These figures show that it is possible to obtain a secretion of inorganic phosphate when sodium phosphate is injected, the urine containing a percentage of 22·5 when the serum level is 10·2, the P output being 1·80 mgr. in 10 minutes, which is about the same as is often registered in an intact anesthetized animal. But the experiment shows, too, that the injection of small amounts of P (150 mgr. in a dog of 9·5 kgr.), raising the serum from 2·8 to 5·0, which is quite a normal percentage, is unable to produce a higher P concentration in the urine than in the serum. It is necessary to reach the abnormal level of 10·2 mgr. in the serum to get a real secretion.

In another experiment (No. 43) where the serum inorganic phosphate was much higher (7·5 mgr.) after removal of the pituitary, the urine containing only 4·2 mgr. P per cent., an injection of 100 mgr. P in form of sodium phosphate raised the serum level up to 9·1 and the concentration in the urine to 15 mgr.

It would seem, then, that after removal of the pituitary or injury of the tuber cinereum the kidney does not lose its ability to secrete inorganic phosphate, as it may put out phosphate in a concentrated form either after administration of organic or inorganic phosphates, but that, in order to obtain a secretion, abnormally high levels of organic or inorganic phosphates are necessary in the serum.

If the normal P secretion is really derived from the inorganic phosphate of the plasma, the only hypothesis which seems to be available to explain the abolition of P secretion is that there is a threshold for P secretion, which is raised by removal of the pituitary or injury to the tuber cinereum. This threshold, which normally seems to be about 5 mgr. or less, would be raised to about 10 mgr. If, on the other hand, we assume that the inorganic phosphate secretion is derived from the organic phosphates of the plasma by means of hydrolysis by the kidney phosphatase, it seems possible that the pituitary or the wall of the third ventricle may regulate the activity of that enzyme by means of a hormone.

Summary and Conclusions.

(1) Removal of the pituitary body or injury to the tuber cinereum may abolish in the following few hours the normal secretion of inorganic phosphates by the kidney.

No one of the experimental conditions can account for this phenomenon.

(2) The change occurring in the kidney secretion is not due to a direct nervous influence, since it appears also in a denervated kidney.

(3) Extract of the anterior lobe of the pituitary body is unable to bring back the phosphate secretion. Pituitrin has inconstant and doubtful effects.

(4) The abolition of phosphate secretion may be dissociated from the experimental polyuria and the lowering of chlorides percentage in the urine.

(5) No change was found in the organic and inorganic phosphate content of the serum sufficient to account for the disappearance of the inorganic phosphate from the urine.

(6) After abolition of the normal phosphate secretion by removal of the pituitary or injury to the tuber cinereum—

- (a) the kidney maintains its ability to concentrate urea, and may still be able to concentrate chloride ;
- (b) it secretes inorganic phosphate when the level of this salt is sufficiently raised in the serum by injection of sodium phosphate, suggesting that there may be a threshold for P secretion, which is raised by the removal of the pituitary or the puncture of the third ventricle ;
- (c) it concentrates and hydrolyses injected organic phosphates, putting out inorganic phosphate in a concentrated form, showing that the kidney enzyme described by Eichholtz, Robison, and Brull is still working, and suggesting that normally a hormone formed in the pituitary or the wall of the third ventricle may regulate the activity of that phosphatase.

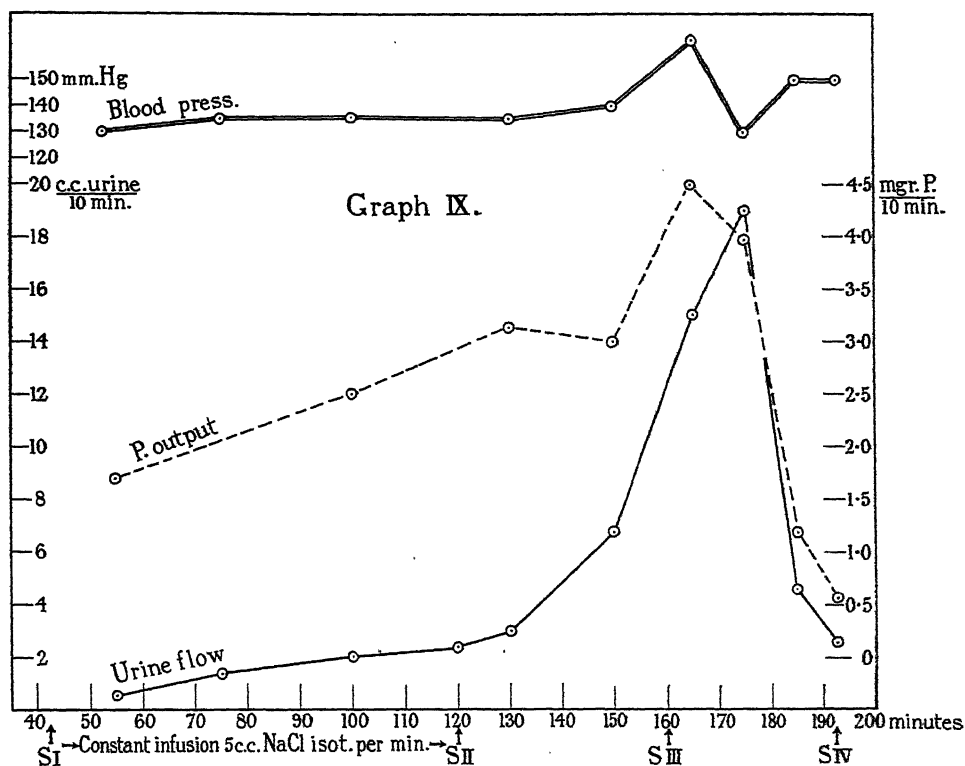
(7) It is suggested that the inability of the isolated kidney, perfused by a heart-lung-kidney preparation, to secrete inorganic phosphate is due to the absence of the regulating mechanism of the pituitary or of the wall of the third ventricle.

APPENDIX.

Influence of Intravenous Injections of Inorganic Salts.

Intravenous infusions of NaCl or KCl, and even of very small amounts of CaCl_2 , generally increase the phosphate output. This increase is accompanied with a larger urine flow, although it does not go up in the same proportion.

Graph IX, Experiment 36, shows that, whereas a constant infusion of isotonic NaCl raises the urine flow from 0.6 to 6.6, the P output is doubled. Further,



	Inorg. P. %	Tot. sol. %
SI		6.81
SII	7.25	5.66
SIII	5.70	5.33
SIV	8.10	4.87

Graph of Experiment 36.

KCl injection produces a copious urine flow, with continued increase of P output, but in a smaller proportion than the urine output.

Ca given afterwards soon decreases the urine flow, but when this flow is still twice as high as the initial one the P output is only one-third of the amount secreted in the beginning.

When Ca is given, together with KCl or separately, it may increase the P output as long as only small amounts are given, but when a certain limit is passed the P secretion falls, and may even be totally abolished. This is shown by the following figures (Table VI) :—

Table VI.—Experiment 26.

Time.	Blood Pressure.	Urine, denervated kidney.			
		Flow cubic centimetres 10 min.	Inorg. P per cent.	Inorg. P mgr. 10 min.	
11.20	120	—	—	—	} 5 c.c. isotonic NaCl per minute.
11.30	110	3.2	150	3.37	
11.45	115	2.0	160	3.20	
12.10	115	4.0	125	5.00	
12.30	110	6.5	80	5.20	
12.45	115	11.3	55	6.20	} 5 c.c. isot. CaCl_2 + KCl per minute (1.5 mgr. Ca, 3 mgr. K per c.c.).
13.00	122	12.3	55	6.70	
13.15	115	19.0	45	8.55	
13.30	70	15.4	2	3.08	
13.45	110	—	—	—	
14.05	120	1.5	0	0	
14.20	110	3.3	0	0	
14.35	—	5.0	0	0	
14.50	120	7.7	0	0	

The decrease of P output is not due to a fall of the level of inorganic phosphate in the serum. On the contrary, the injection of Ca is generally followed by an increase of serum P. This is shown by the figures of Experiment 36 (Graph IX), where the fall of urinary P was accompanied by an increase in the serum from 5.7 to 8.1.

Summary.

(1) Infusions of NaCl or KCl solutions increase the output of inorganic phosphate together with the urine flow, but in a smaller proportion.

(2) Small amounts of CaCl_2 may increase the P output; large amounts diminish or even abolish inorganic phosphate secretion. This decrease of

phosphate in the urine is generally accompanied by an increase of the phosphate level of the serum.

We express our indebtedness and thanks to Prof. E. H. Starling for his continuous advice and his suggestions during the performance of this work.

REFERENCES.

- (1) L. Brull and F. Eichholtz, 'Roy. Soc. Proc.' (*preceding paper*).
- (2) A. P. Briggs, 'Jnl. Biol. Chem.', vol. 53, p. 13 (1922).
- (3) Millard Smith, *ibid.*, vol. 45, p. 437 (1921).
- (4) J. Camus et G. Roussy, 'Jnl. de Phys. et Path. gén.', vol. 20, pp. 509 and 535 (1922).
- (5) Eichholtz, Robison and Brull (*following paper*).

Hydrolysis of Phosphoric Esters by the Kidney in Vivo.

By F. EICHHOLTZ (Rockefeller Travelling Fellow), ROBERT ROBISON and
L. BRULL.

(Communicated by E. H. Starling, F.R.S., Foulerton Professor of the Royal Society.
—Received August 6, 1925.)

(From the Physiological Institute, University College, London, and the Biochemical
Department, Lister Institute, London.)

Robison (1923) found that aqueous extracts of macerated kidney contain an enzyme which hydrolyses phosphoric esters, such as hexosephosphates and glycerophosphate. This enzyme is also present in bones and teeth, and occurs in cartilage as soon as ossification starts. It is also present in the intestinal mucosa, but other tissues contain it in traces only. It is characterised by a high optimum p_H (8.4–9.4). At p_H 9.3 its activity is five times as great as at p_H 7.3 (Robison and Soames, 1924). It was suggested by Robison that this enzyme plays an important rôle in the calcification of bone, and the suggestion has been supported by a considerable amount of evidence (3, 4, 5). Nothing, however, was known of the function of the enzyme in the kidney. It was thought possible that it might be required for the eventual hydrolysis and excretion of those phosphoric esters, which are present in considerable amount in the red corpuscles and in small amount in the plasma.

Eichholtz and Starling (1925) have recently shown that the isolated kidney when perfused by means of a heart-lung preparation, does not excrete inorganic

phosphates. This disability they attribute to the fact that the inorganic phosphates of the serum are present in a state to which the glomerular membrane is impermeable. They suggest, therefore, that the normal urinary phosphates are secreted by the tubule cells. Moreover, under the conditions of the experiment, the phosphate excretion would seem to be more readily extinguished than the excretion of either urea or sulphate.

Brull and Eichholtz (7), from experiments on the whole animal, have come to the conclusion that the excretion of phosphate is readily affected or even abolished by extirpation of the pituitary gland or injury of the tuber cinereum. These experiments have shown, moreover, that the extinction of phosphate excretion is not brought about by any abnormal variation of the concentration of inorganic phosphates in the serum.

The present investigation of the fate of phosphoric esters when introduced into the circulation was undertaken in the hope of gaining some insight into the significance of the presence of this enzyme in the kidney. For this purpose Starling's heart-lung-kidney preparation was employed, the technique of which has been described in previous papers by Starling and Verney, Eichholtz and Starling (6, 8).

Since the precise nature of the phosphoric esters in the blood is not yet known, sodium glycerophosphate was used. This ester and hexosemonophosphate are hydrolysed at about the same rate by kidney extracts. The ester was added to the defibrinated blood in the form of a M/8 solution. Inorganic and organic acid-soluble phosphates in urine and serum were estimated by the Briggs' method, using the precautions noted by Martland and Robison (1924).

The first series of our experiments showed *that the isolated kidney, when perfused with blood containing added glycerophosphate, hydrolysed the phosphoric ester* to a very notable extent, so that the urine secreted by the kidney contained not only organic but also inorganic phosphates in considerable quantity. The amount of hydrolysis, however, varied considerably, and in some of the later experiments scarcely any hydrolysis occurred.

Details of two experiments exemplifying these different results are given in Table I below.

The results show a 6-8 fold concentration of glycerophosphate in the urine as compared with the serum values, and a large increase of inorganic phosphate, apparently indicating that about 10 per cent. of the ester has been hydrolysed during its passage through the kidney. The figures further suggest that the concentration of inorganic phosphate was rapidly increasing, and that later samples might have shown evidence of a much higher degree of hydrolysis.

Table I.—Experiment II. Heart-lung-kidney preparation—normal kidney connected at 0' together with nephritic kidney at 10' (subcutaneous injection of 1.0 gm. per kilog. of tartaric acid, racemic form, two days previously). 2.0 gm. urea + 1 c.c. adrenaline, 1:100,000, added to circulating blood. Weight of normal kidney 42.0; weight of nephritic kidney, 30.0. At 91' sodium glycerophosphate equivalent to 200 mgr. P was added to the blood. The concentrations of organic and inorganic phosphates are given in milligrammes P per 10 c.c.

Mean Time after connection. Min.	Temperature ° C. of Blood at Kidney.		Blood Pressure mm. Hg. at kidney.	Renal Blood Flow, cubic centimetres. Mins.		Serum.		Urine, Normal.				Urine, Nephritic.			
	Normal.	Nephritic.		Normal.	Nephritic.	Org. P.	Inorg. P.	Flow cubic centimetres. 10 min.	Protein.	Org. P.	Inorg. P.	Flow cubic centimetres. 10 min.	Protein.	Org. P.	Inorg. P.
65	34.5	34.0	104	188	72	—	—	10.0	+	—	0.2	—	—	—	—
80	35.0	35.0	104	200	85	3.0	—	8.0	+	—	0.2	—	—	—	—
90	35.0	35.0	105	187	78	—	—	9.1	+	4.8	0.2	0.6	+	3.3	11.9
100	36.0	35.5	108	150	67	—	—	8.0	+	—	—	1.5	+	—	—
110	36.0	35.5	108	136	52	30.7	4.8	—	—	—	—	—	+	—	—
112	—	—	—	—	—	—	—	6.0	+	223	13.1	—	+	—	—
120	36.0	35.0	108	120	43	—	—	4.0	+	217	25.8	1.2	+	149	51.7
130	—	—	—	—	—	19.8	8.5	—	—	—	—	—	—	—	—

Table II.—Experiment VII. Heart-lung-kidney preparation—Kidney connected at 0'. 2.0 gm. urea + 1 c.c. adrenaline 1 : 100,000 added to circulating blood. Weight of kidney, 38.0 gm. At 67' sodium glycerophosphate equivalent to 200 mgr. P added to blood. The concentrations of organic and inorganic phosphates are given in milligrammes P per 100 c.c.

Mean time, minutes.	Temp. of blood at kidney, 0° C.	Blood pressure at kidney (mm. Hg.).	p_H of arterial blood.	Renal blood flow, cubic centi- metre/ min.	Serum.		Urine.			
					Org. P.	Inorg. P.	Flow, cubic centi- metres, 5 min.	Pro- tein.	Org. P.	Inorg. P.
	Brea	thed air.	Urine	starts.						
5	—	—	—	—	—	—	—	—	—	—
54	35.5	109	—	143	0	2.6	—	—	—	—
59	35.5	109	—	143	—	—	2.5	—	0	2.5
64	35.5	109	—	143	—	—	2.4	—	0	0.9
67	—	—	—	—	—	—	—	—	—	—
79	35.5	109	—	177	—	—	2.8	—	199	1.1
84	35.8	109	7.65	177	19.7	3.6	3.2	—	190	1.8
	Breat	hed 8.4	per cent.	CO ₂ .						
94	35.0	108	7.32	188	16.5	5.3	2.9	—	147	2.8
99	35.0	109	—	188	—	—	3.0	—	172	1.3

The results show a 10-fold concentration of glycerophosphate in the urine but no appreciable hydrolysis.

In another experiment in which a similar result was obtained, an extract prepared from the perfused kidney was found to possess very little hydrolytic power towards sodium glycerophosphate. It seemed, therefore, to be possible that a negative hydrolysis might be explained by a deficiency of enzyme in the perfused kidney or by its inactivation as a result of the perfusion. Other controls showed, however, that this was not the case, and that a perfused kidney which does not hydrolyse organic phosphates, may contain at the end of the experiment a normal amount of enzyme. Since the kidneys, which were unable to split up the added organic phosphates, showed other signs of secretory deficiency, notably a very poor urine output as compared to the average, it is probable that the negative results were due to occasional deficiency of the perfusion method.

The following facts have emerged from our experiments, examples of which have just been set out. The addition of sodium glycerophosphate in M/8

solution, equivalent to 100–200 mgr. P to 600–1000 c.c. of circulating blood, had practically no effect on either blood or urine flow.

The concentration of organic acid-soluble phosphate in the serum, which was at first raised from values between 0.5–1.8 mgr. to 15–25 mgr. P per 100 c.c., gradually fell as the experiment proceeded, the ester being partly replaced by inorganic phosphate, which increased from 2.5–3.5 mgr. to 6.1–8.5 mgr. P per 100 c.c. (in one experiment this value rose to 12.5 mgr. in 2 hours).*

After the addition of glycerophosphate, there was always a high concentration of organic phosphates in the urine compared with that in the serum. Thus, in six experiments the values of this ratio were 3.4, 12.5, 11.5, 7.2, 15.2, 9.8.

The concentration of organic phosphates in the urine gradually diminished as the experiment proceeded. In some experiments there was a sudden fall in the excretion which could not be explained by any equivalent decrease in the concentration of phosphoric ester in the serum.

It was known from previous experiments, and has been confirmed in the present series, that during perfusion of the isolated kidney there is a gradual falling-off in the excretion of urea, and this has been explained as due to the increasing failure of the secretory powers of the kidney. It is suggested that the falling-off in the excretion of glycerophosphate is also due in part to this failure of secretory power, as well as to the disappearance of the ester from the serum.

In the majority of cases part of the glycerophosphate was hydrolysed during its excretion by the kidney. After the addition of glycerophosphate to the blood the excretion of inorganic phosphates did not at once rise to a maximum but increased gradually throughout the experiment. The concentration of inorganic phosphates in the urine might thus rise to 10–20 times the concentration in the serum, and in some cases amounted to more than 90 per cent. of that of the total urinary phosphates.

Eichholtz and Starling (6) have shown that in the heart-lung-kidney preparation the inorganic phosphates of the serum do not pass the glomerulus. In their experiments, when additional inorganic phosphates were introduced into

* The low figures for the initial concentration of inorganic phosphate in the blood suggest that synthesis of phosphoric esters had taken place during defibrination owing to the increased alkalinity (Martland, Hansmann and Robison, 10, Martland, 11). Whether the rise occurring after addition of glycerophosphate was in any part due to the kidney cannot be decided, as the venous and arterial blood were not separately examined.

the circulating blood these might appear in the urine for a short time, but the concentration in the urine never exceeded that in the serum, when the figures for the latter had been duly corrected for the volume of the serum protein.

This statement was not entirely confirmed in the present series of experiments. In two cases the injection of 50 mgr. P in form of sodium phosphate was not followed by a secretion of inorganic P by the kidney, but in another similar experiment the same injection produced an excretion of inorganic phosphates in a concentrated form.

Further, Brull and Eichholtz (*loc. cit.*) found that when the P secretion is abolished in the whole animal by removal of the pituitary or puncture of the tuber cinereum, it is possible to produce a secretion of inorganic phosphates by intravenous injection of sodium phosphate, the concentration in the serum being slightly increased (for instance, by raising the serum P from 2.8 to 10.2 the concentration in the urine goes up from 0 to 24 in the urine).

The possibility that the appearance in the urine of inorganic P in a concentrated form after injection of organic P might be due to a small increase of inorganic P in the serum, observed in some experiments, was therefore examined.

Three perfusion experiments were performed in which sodium phosphate was injected, followed after a certain period by glycerophosphate.

In one experiment the addition of sodium phosphate produced no secretion of phosphates. A small excretion, both of inorganic and of organic phosphates, followed the addition of glycerophosphate, but the urine flow was so unsatisfactory that one cannot draw any conclusion.

The results of the two other experiments are shown in Tables III and IV.

In the first of these (Table III) the addition of inorganic P raised the concentration of inorganic phosphates in the serum from 2.18 to 5.58, but caused no excretion. The further addition of glycerophosphates was followed by a marked secretion, the urinary concentration rising to 57.7 mgr. per hundred cubic centimetres. The concentration of inorganic P in the serum, however, simultaneously rose to 7.36, which might account for the rise in the urine phosphates if a threshold is postulated.

The figures of the third experiment (Table IV), while supporting in some respects the hypothesis of a threshold, in that a rise in the serum concentration of inorganic phosphates from 4.0 to 7.8 was accompanied by a notable increase in phosphate excretion, supply definite evidence that the secretion of inorganic phosphates following the addition of glycerophosphates is not due to such a cause. At a time when the phosphate's excretion is falling, injection of 50 mgr.

Table III.—Heart-lung-kidney preparation. Kidney connected at 11 h. 50. —
3 gr. urea and 1 c.c. adrenaline 1/100,000 added to circulating blood.
Weight of kidney, 42.5 gr.

Time.	Temp. blood at kidney.	Blood pressure (mm. Hg.).	Renal blood flow, cubic centi- metre/ min.	Serum.		Urine.			
				Org. P.	Inorg. P.	Flow, cubic centi- metres, 10 min.	Pro- tein.	Org. P.	Inorg. P.
11.50	37.0	98	78	—	—	—	—	—	—
12.05	—	—	—	—	—	2.06	+	0.6	36.7
12.12	36.5	102	100	0.47	2.18	—	—	—	—
12.15	—	—	106	—	—	5.50	+	0.8	2.53 50 mgr. P (so- dium phosphate)
12.30	36.0	102	107	—	—	4.00	+	0.2	1.57
12.35	—	—	—	0.57	5.58	—	—	—	—
12.40	36.5	103	120	—	—	8.40	+	0.24	0.90
13.00	37.0	105	166	—	—	9.70	+	0.10	0.70
13.15	37.0	106	161	0.27	5.04	—	—	—	—
13.20	—	—	—	—	—	8.00	+	0.15	0.73 50 mgr. P (gly- cero- phosphate)
13.27	—	—	—	7.66	5.19	—	—	—	—
13.30	37.0	106	166	—	—	5.60	+	13.2	1.11
13.50	36.0	106	150	—	—	3.50	+	33.8	8.67
14.10	36.5	—	—	—	—	3.45	+	19.0	24.70
14.30	36.5	106	130	—	—	1.30	+	11.3	37.0
14.40	37.0	106	130	2.24	7.36	—	—	—	—
15.10	36.5	100	100	—	—	0.50	+	11.3	57.70

of organic phosphorus produces a largely increased output, although the serum level was continuously falling.

In this experiment the ratio of inorganic to organic P in the urine rose to 15/13 with a serum concentration of 5.6 mgr. inorganic and 1.9 organic P, which corresponds with 90 per cent. hydrolysis.

There is further possibility that the phosphate excretion which sometimes followed the addition of inorganic phosphate may itself have been due to small increases in the concentration of phosphoric esters in the plasma owing either to synthesis or to displacement of the equilibrium between corpuscles and plasma. We have at present no experimental evidence on this point.

If a P threshold exists, it would in these experiments appear to be much higher than the normal level of inorganic phosphates in the living animal.

There seems therefore no doubt, that even though the kidney is able to

Table IV. See also Chart I. Heart-lung-kidney preparation. Kidney connected at 12 h. 10, 3 gr. urea and 1 c.c. adrenaline 1/100,000 added to circulating blood.

Time.	Temp- erature of Blood at Kidney.	Blood Pressure mm. Hg.	Renal Blood Flow. Cubic centi- metres per min.	Serum.		Urine.			
				Org. P.	Inorg. P.	Flow cubic centi- metres per 10 min.	Pro- tein.	Org. P.	Inorg. P.
h. m.									
12.10	37.0	120	14	—	—	—	—	—	—
12.21	—	120	50	—	—	starts	—	—	—
12.35	37.5	110	100	—	—	3.6	+	0.03	8.0
12.42	—	—	—	1.2	4.0	—	—	—	—
12.45	38.0	110	125	—	—	8.5	+	0.00	2.0. 50 mgr. in- org. P (sodium phosphate).
12.50	37.0	105	—	—	—	10.0	+	0.20	4.0
12.55	—	100	130	1.4	7.8	10.4	+	0.00	30.0
13.00	37.0	108	150	—	—	12.6	+	0.00	24.0
13.05	—	100	—	—	—	10.2	+	0.00	21.9
13.10	36.0	100	150	—	—	14.2	+	0.00	19.6
13.15	36.0	102	150	0.8	7.2	14.8	+	0.00	17.9. 50 mgr. org. P. (glycerophos- phate.)
13.20	—	100	166	—	—	17.0	+	0.60	17.4
13.22	—	—	—	10.0	7.2	—	—	—	—
13.25	36.0	100	166	—	—	18.0	+	27.4	32.4
13.30	—	—	—	—	—	19.0	+	20.9	34.0
13.35	—	100	176	7.5	6.6	17.2	+	16.7	34.0
13.40	35.5	100	—	—	—	17.0	+	15.1	34.9
13.52	—	100	176	—	—	15.0	+	11.2	35.0
14.07	36.0	97	—	—	—	11.4	+	6.0	38.8
14.15	—	98	176	3.4	6.2	9.6	+	5.7	42.2. 250 c.c. blood added.
14.25	36.0	100	—	1.9	5.6	—	—	—	—
14.30	—	100	160	—	—	3.0	+	5.0	57.0
14.40	—	—	176	—	—	4.0	+	3.0	44.3. 100 mgr. org. P. (glycerophos- phate.)
15.53	34.0	100	136	25.7	6.0	—	—	—	—
16.30	—	120	176	—	—	0.9	+	116.2	69.3

concentrate injected inorganic P, the *inorganic P which appears in the urine in a concentrated form after administration of organic phosphoric esters, depends on the hydrolysis of these esters in the kidney, and not on an eventual small increase of inorganic P in the serum.*

Further Evidence on the Place where the Hydrolysis Occurs.

The question arises now whether the urine itself contains the enzyme, and whether the hydrolysis does not occur *after* the secretion of the ester and during the passage of the urine down the tubules.

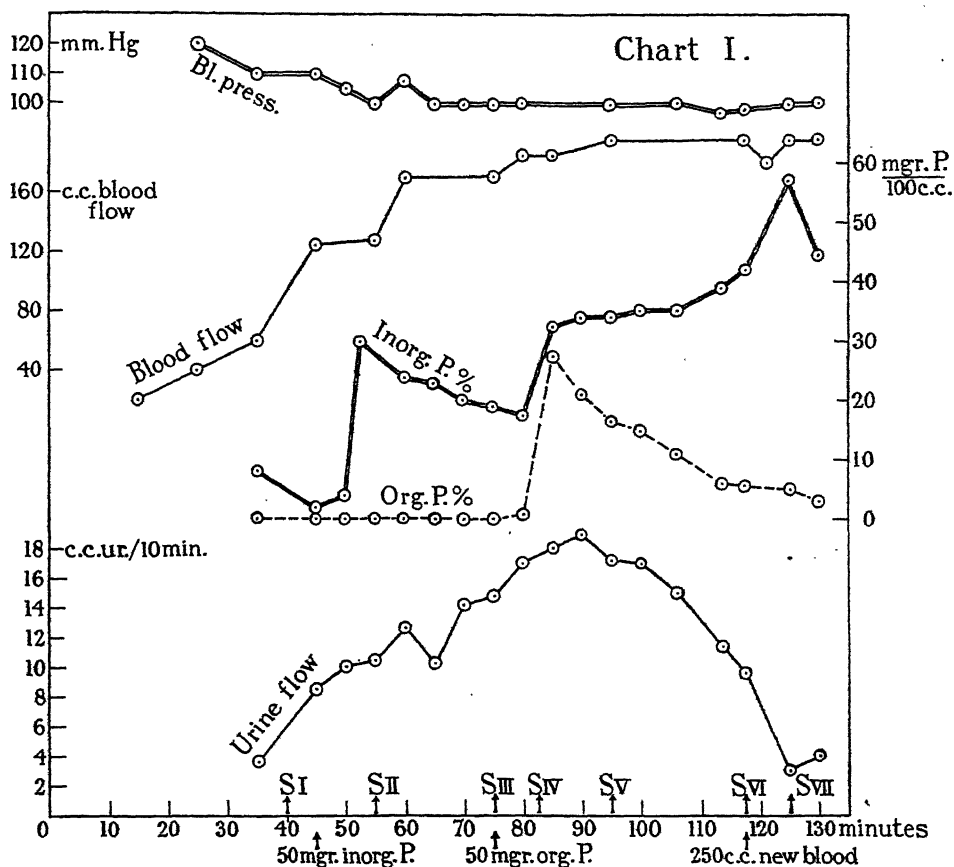


CHART I.

	Serum phosphates. P (mg.) per 100 c.c. serum.						
	S ₁ .	S ₂ .	S ₃ .	S ₄ .	S ₅ .	S ₆ .	S ₇ .
Inorganic	4.0	7.8	7.2	7.2	6.6	6.2	5.6
Organic	1.2	1.4	0.8	10.0	7.5	3.4	1.9

Tests were therefore carried out on various samples of the experimental urine by measuring the amount of hydrolysis of sodium glycerophosphate effected by 1 c.c. of the urine in 18 hours at 38 degrees, at p_H 7.4 and 8.4 (v. Robison and Soames, 2). Hydrolysis was observed in some cases, showing that the enzyme was present in small but definite amount. These samples

also contained protein so that the enzyme may have been derived from the breaking down of the tubule cells. Similar results have been obtained with human urine, normal samples containing none, or only traces of the enzyme, while samples from nephritic patients may contain appreciable amounts.

The results of a number of these tests are given in Table V, the amounts of hydrolysis being calculated for 100 c.c. urine.

Although, therefore, there is no doubt that under certain conditions the enzyme may appear in the urine, the actual amount is always very small, and could not account to any appreciable extent for the hydrolysis of glycerophosphate observed in the perfusion experiments. This hydrolysis must therefore take place in the kidney cells themselves.

Table V.

Sample of Urine.	Protein.	Sodium glycerophosphate (mg. P) hydrolysed in 18 hrs. at 38° by 100 c.c. urine.	
		$p_H = 7.4$	$p_H = 8.4$
Experiment II—			
Normal kidney....	+	—	12
Nephritic kidney....	+++	—	99
Experiment III—			
Living dog immediately after denervation of kidney....	++	20	53
Same dog later....	trace	0	7
Same dog later....	0	0	2
Normal human....	0	1	1
Normal human....	0	3	3
Nephritic human....	++	6	18

Effect of Urinary Flow on the Extent of Hydrolysis.

Although the hydrolysis is not determined by the enzyme which is washed out of the kidney, it might be supposed that it takes place in the lumen of the tubules by contact of the organic ester with the surface of the cells lining the tubules. In this case an increased urinary flow should wash out the ester in a shorter time so that the proportion hydrolysed should be diminished. The following experiment demonstrates that this is not the case.

Table VI. Experiment VI. (Chart II). Heart-lung-kidney preparation. Kidney connected at 0'. 3 gm. urea + 1 c.c. adrenaline 1 : 100,000 added to circulating blood. Weight of kidney, 38.5 gm.

Urine as it was secreted was directly dropped into trichloroacetic acid to prevent any enzyme action.

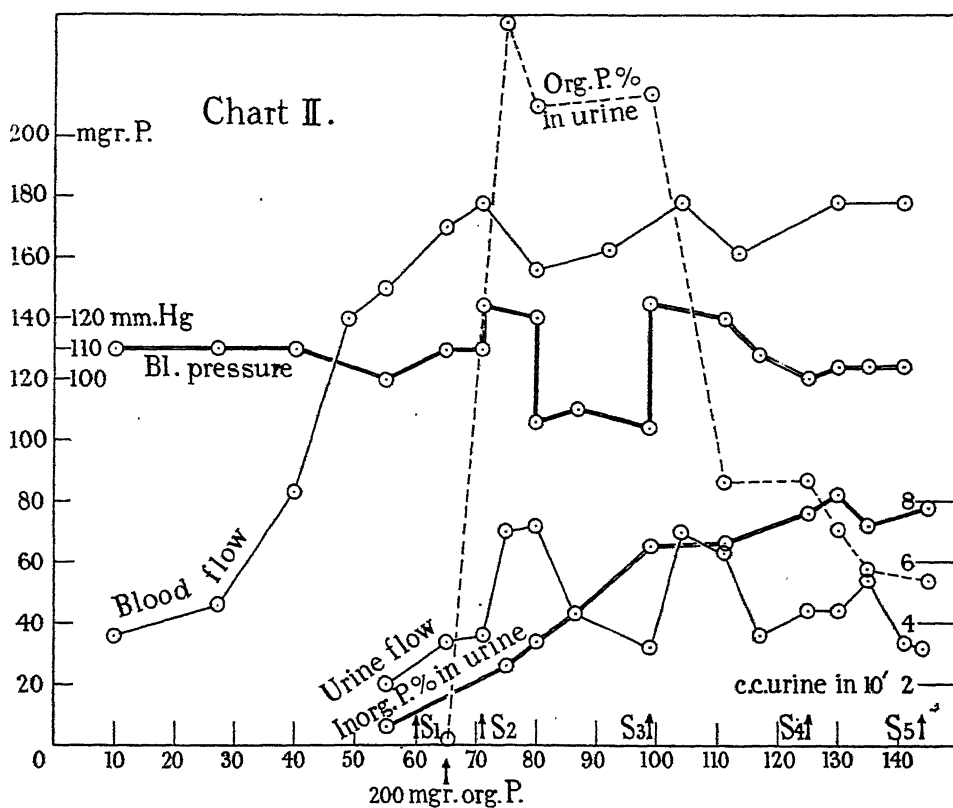


CHART II.

	P (mg.) per 100 c.c. serum.				
	S ₁ .	S ₂ .	S ₃ .	S ₄ .	S ₅ .
Inorganic	3.5	4.3	6.4	7.4	8.4
Organic	1.8	24.2	15.5	11.6	6.2

At 65' sodium glycerophosphate equivalent to 200 mg. P was added to the circulating blood.

The results are shown graphically in Chart II, and are summarised in the following table :—

Table VI.

Blood pressure at kidney (mm. Hg.).	Urine flow, cubic centimetres/10 min.	Conc. in urine. Conc. in serum.		Total excretion per 10 min. mg. P.		Inorganic phosphates in percentage of total phosphates in urine.
		P Org.	P Inorg.	Org.	Inorg.	
120	7	9.5	6.8	13.7	2.6	15.8
85-90	3-4	12.5	10.3	6.8	2.3	25.4
120	6-7	5.3	9.6	5.5	4.2	43.5

The figures show that whereas the excretion of organic phosphate diminishes that of inorganic phosphate increases, and thus the percentage of total urinary phosphates excreted in inorganic form steadily rises. There is, therefore, no indication that the extent of hydrolysis is governed by the time during which the ester remains in the tubules after being secreted by the cells.

This points to the conclusion that hydrolysis occurs within the cells, and like the secretion is independent of the rate of urinary flow.

Effect of the Reaction of the Blood.

It has been shown that the optimum p_H of the phosphoric esterase which is present in bones and in the kidney lies between 8.4 and 9.4, and that the activity diminishes rapidly towards p_H 7.0. If, therefore, the hydrolysis observed in these experiments with the isolated kidney was indeed brought about by this enzyme, the lowering of the p_H of the blood by breathing CO_2 should cause a reduction in the amount of hydrolysis.

In four experiments CO_2 was supplied to the heart-lung-kidney preparation, so that the p_H of the circulating blood changed from about 7.50-7.60 to 7.20-7.30. From the results of these experiments it was evident that a decrease in the rate of hydrolysis of organic phosphates occurred during CO_2 breathing, and the supposition that the hydrolysis is due to the enzyme appears to be justified. An example of the CO_2 effect is shown in Chart III.

Chart III. Experiment X.—Heart-lung-kidney preparation. Kidney connected at 0'. 3 gm. urea+1 c.c. adrenaline 1:100,000 added to circulating blood. Weight of kidney 31.5 gm.

At 38' sodium glycerophosphate equivalent to 100 mg. P was added to the blood.

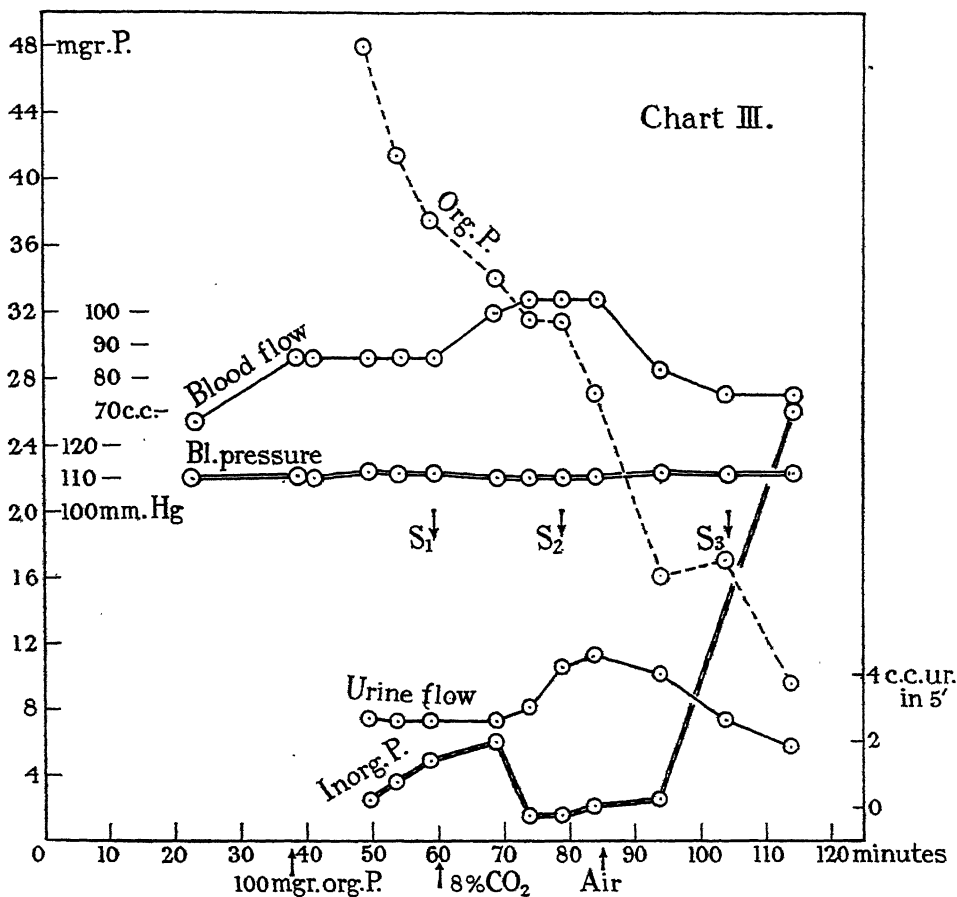


CHART III.

	P (mg.) per 100 c.c. serum.		
	S ₁	S ₂	S ₃
Inorganic	2.2	1.4	2.6
Organic	10.2	11.2	7.6

Experiments on the Whole Animal.

Brull and Eichholtz have shown that it is possible to abolish the phosphate excretion in the whole animal by cutting out the pituitary, or puncturing the tuber cinereum. If phosphates are lowered by this operation they do not recover again in the next few hours, but tend to disappear completely. In an animal in which the phosphate excretory function has been damaged in this way it is possible to investigate quantitatively the fate of injected organic phosphates. The following experiment (XII) may be given (see p. 105).

It is evident that after injection of glycerophosphate into the whole animal there is a large excess of organic phosphate in the serum, rising from 0.5 to 3.55. This organic phosphate behaves as a foreign substance, and as such is concentrated and excreted by the kidney.

The added phosphoric ester disappears from the serum very rapidly, only a trace being present in a sample taken 30' after injection, and the amount being practically at the normal level in 60'. As the organic phosphate in serum diminishes, an ever-increasing secretion of inorganic phosphate takes place, so that 40' after injection the whole of the phosphates are coming through in inorganic form.

In the same time there is a slight increase of inorganic phosphates in the serum, which rise from 4.3 to 5.9, falling in the next samples to 5.0. This increase is absolutely insignificant against the enormously increased urinary phosphates.

During one hour after the injection of 60 mg. P as glycerophosphate, 10 mg. P were excreted in organic form with the first samples of urine, while 35 mg. P were excreted as inorganic phosphate.

When we compare the behaviour of the kidney *in vivo* with that of the heart-lung-kidney preparation, we see that, in the latter, the degree of hydrolysis of the glycerophosphoric ester never reaches the same level as that attained in the whole animal. In this respect the isolated kidney behaves to phosphates as it does to urea, sulphate or phenolphthalein. Occasionally, however, this power of splitting organic phosphate may be found entirely absent in a heart-lung-kidney preparation, which yet reacts normally as regards chlorides, urea and sulphates.

The significance of the high optimum p_H of the enzyme remains here, as in the case of the bones, a highly interesting but still unsolved problem; but these experiments prove that at the normal p_H of the blood, whatever may be the p_H of the kidney cell fluids under such conditions, the enzyme is able to display considerable activity.

Chart IV. Experiment XII.—Bitch, 8.7 kg. 2 years old. Left kidney denervated from the back. Cannulas inserted in both ureters by retro-peritoneal operation.

11.32.—Collection of urine started.

11.32–11.56.—Pituitary cut out. 60 c.c. of saline given intravenously. 103' after cutting out pituitary, glycerophosphate equivalent to 60 mg. P in M/8 solution was injected intravenously.

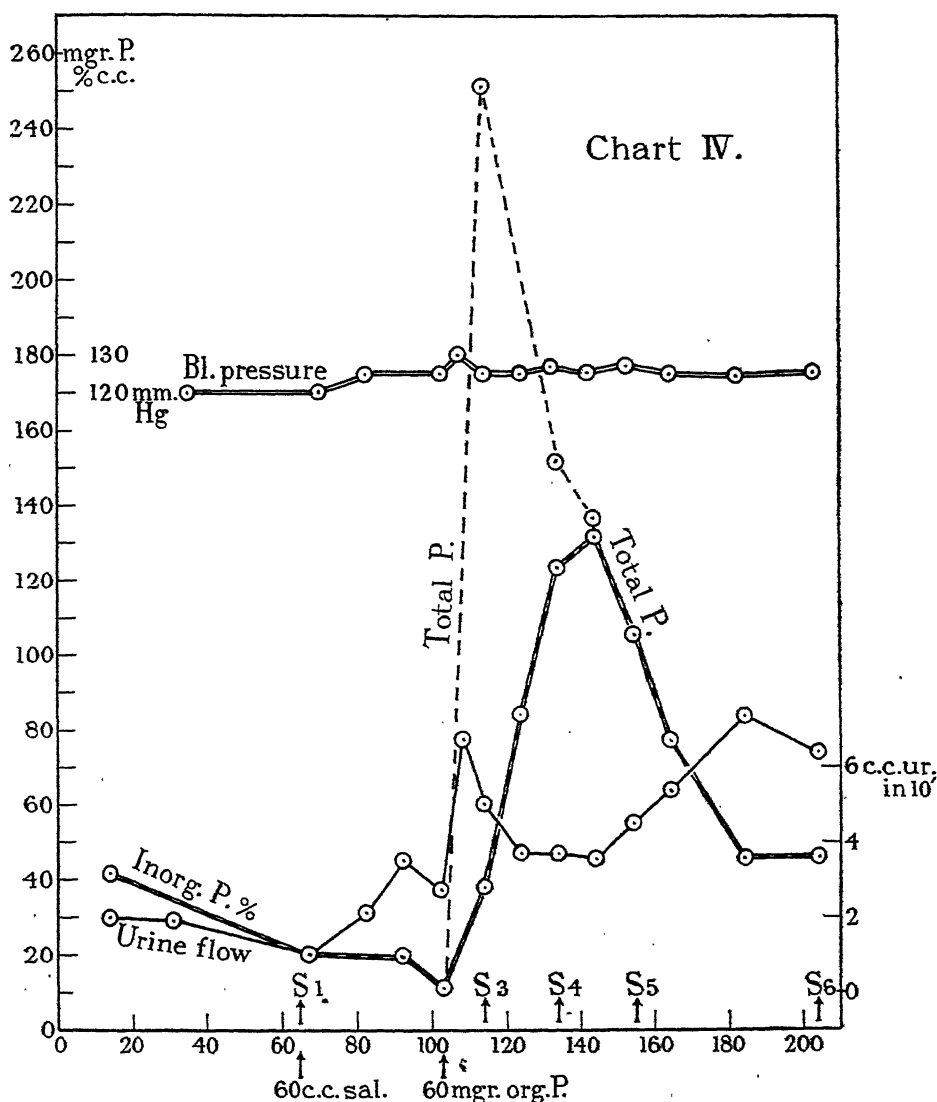


CHART IV.

	P (mg.) per 100 c.c. serum.					
	S ₁ .	S ₂ .	S ₃ .	S ₄ .	S ₅ .	S ₆ .
Inorganic	4.2	4.3	5.2	5.9	5.0	5.0
Organic	—	0.5	3.5	0.6	0.5	0.5

Conclusions.

The isolated kidney, perfused by means of the heart-lung preparation, which is unable to concentrate the normal inorganic phosphates of the blood serum, is able to concentrate and to hydrolyse added organic phosphates, and to excrete their phosphorus as inorganic phosphates in the urine in a much higher concentration than that of the inorganic phosphates in the serum.

In the whole animal in which the urinary inorganic phosphates have sunk to a minimum after removal of the pituitary or puncture of the tuber cinereum, injected organic phosphates are excreted, after an initial stage, entirely in inorganic form.

The suggestion is put forward that normally a considerable part, if not all, of the urinary phosphates are derived from the organic phosphates of the serum by a process of hydrolysis in the cells of the kidney under the action of the kidney enzyme.

This work was carried out in the laboratory of Prof. Starling, F.R.S., for whose continuous interest and help we express our indebtedness and gratitude.

REFERENCES.

- (1) Robison, 'Biochem. Jl.,' vol. 17, p. 286 (1923).
- (2) Robison and Soames, 'Biochem. Jl.,' vol. 18, p. 740 (1924).
- (3) Kay and Robison, 'Biochem. Jl.,' vol. 18, p. 755 (1924).
- (4) Goodwin and Robison, 'Biochem. Jl.,' vol. 18, p. 1161 (1924).
- (5) Martland and Robison, 'Biochem. Jl.,' vol. 18, p. 1354 (1924).
- (6) Eichholtz and Starling, 'these Proceedings,' B, vol. 98, p. 93 (1925).
- (7) Brull and Eichholtz, 'these Proceedings' (*preceding paper*).
- (8) Starling and Verney, 'these Proceedings,' B, vol. 97, p. 321 (1925).
- (9) Martland and Robison, 'Biochem. Jl.,' vol. 18, p. 765 (1924).
- (10) Martland, Hansmann and Robison, 'Biochem. Jl.,' vol. 18, p. 1152 (1924).
- (11) Martland, 'Biochem. Jl.,' vol. 19, p. 117 (1924).

Address of the President, Sir Charles Sherrington, O.M., at the Anniversary Meeting, November 30, 1925.

Let me in the first place refer, even if briefly, to those whom death in the past year has removed from the roll of the Fellowship. The losses this year have been numerous. I follow the order of their occurrence.

GEORGE DOWNING LIVEING, chemist, who held the Chair in Cambridge from, if we include the term of his deputy tenure, 1859 until 1908, and witnessed his University pass, largely owing to his own influence, from a stage with hardly any provision for the practical teaching of chemistry to a development of it such as pertains to the full modern scale of requirement. His original contributions to his science lay largely in spectroscopy. Part of his personality was a vigour, bodily and mental, retained into advanced age, for he had reached his 98th year. He had been throughout his career a force in his University. He was awarded the Davy Medal in 1901.

WILLIAM WHITAKER, geologist, and in the service of the Geological Survey for 39 years. He continued to render unofficial help to the Geological Survey almost up to the extreme end of his long life of 88 years.

WILLIAM AITCHISON HASWELL, zoologist, Professor of Biology in the University of Sydney. An untiring investigator in the field of comparative anatomy; his varied work included the description of a number of remarkable animal forms which he was the first to record. His latest paper, published only last December, amplified a monograph on the Temnocephalæ that he had published thirty years before. He was joint author with Geoffrey Parker of a large text-book of zoology, of standard reference throughout the English-speaking world.

JAMES MACKENZIE, physician, specially distinguished for the advances he made in, and the impetus he gave to, modern clinical study of diseases of the heart, work begun and largely completed amid the pre-occupations of a general medical practice at Burnley. There he devised his polygraph for recording simultaneously the arterial and venous pulses to gauge the action of the heart's auricle, a key to the understanding of disturbed rhythm of the heart. In 1907 he came to London and devoted himself to consultant work with great success. Finally, he removed to St. Andrews, where his name will always be connected with the Institute for clinical study which he there founded.

OLIVER HEAVISIDE, mathematical physicist. Withdrawing in early life from business as a telegraph engineer, he devoted himself in retirement to problems of electricity and of its applications. It has been written of him that he, perhaps more than any other one man, proved the value of mathematical theory to the electrical industry. He was elected a Fellow of the Society in 1891. Two years later appeared the first volume of his 'Electro-Magnetic Theory'; the third and concluding one in 1912. He described what is now familiar to the radio engineer as the Heaviside layer, by which, it is supposed, Hertzian waves are bent round the earth. He was a nephew of Charles Wheatstone.

HORACE TABBERER BROWN, chemist and botanist. Largely self-taught in natural science, though having received some formal instruction in chemistry, he entered a brewery, and at once recognized the scope for microscopic and chemical study of yeasts, and of the then novel cognate studies of Pasteur on Vinous Fermentation. It has been penetratively remarked that he achieved for brewing improvement and success analogous to those achieved for surgery by Lister. He further contributed fundamental researches on the transformation and shift of the reserve carbo-hydrate in germinating grain; also on changes in the green leaf during photosynthesis, and on the amount of absorption of carbon dioxide through the stomata in relation to gaseous diffusion. He received a Royal Medal in 1903 and the Copley Medal in 1920.

EDWIN KLEIN, histologist and bacteriologist, for many years a teacher in the Medical School of St. Bartholomew's Hospital, and an investigator, mainly bacteriological, for the Medical Department of the Local Government Board. He had been a Fellow of the Society for close on 50 years.

THOMAS CLIFFORD ALLBUTT, physician, Regius Professor of Physic at Cambridge from 1902 until his death, in his 89th year. By character and attainments, an outstanding figure in Medicine throughout his long career. Of Yorkshire family, the first 27 years of his professional life were spent in Leeds, and in that period he helped to bring into general clinical use the sphygmomanometer for measuring blood pressure; and he introduced the clinical thermometer in its present practical form. At Cambridge, he was not only the titular head, but the life and soul of the Medical School. He did much public work for the organization of Medical education throughout the country. He was made a Privy Councillor in 1920. An accomplished writer, there can hardly be a more charming example of him

as such than the obituary notice of his old friend Pridgin Teale, which he furnished for the Proceedings of this Society two years ago. He was a Vice-President of the Society from 1914 to 1916.

EDWARD THORPE, chemist, distinguished in research, in teaching and in administration. His accurate measurements of specific volumes of liquids of related chemical constitution, led him to conclusions which at the time were novel. His researches with Dr. Tutton into the oxides of phosphorus brought among other results the discovery of the tetroxide, also incidentally the recognition of the oxide (P_4O_6) as the cause of the jaw-necrosis of match-factory workers, a recognition which enabled the stamping-out of the disease. The atomic weight of radium was the subject of his Bakerian Lecture in 1907. He will be remembered also in connection with the Magnetic Survey of the British Isles. Professor of Chemistry at Leeds, and then at South Kensington, and then Government Chemist, he returned to South Kensington as Professor of General Chemistry in the Imperial College of Science and Technology, and retired at the end of the war. He was foreign Secretary of the Society from 1899 to 1903.

JOHN CLELAND, anatomist, at first of Queen's College, Galway, and then for 32 years in the Chair of Anatomy at Glasgow. His original contributions were of considerable volume, but his chief impression upon his time was as a teacher.

GEORGE NATHANIEL CURZON, Marquess Curzon of Kedleston, elected to the Society under Statute 12 in 1898, had travelled extensively in Asia. Was Viceroy and Governor-General of India from 1899 to 1905. A great public servant in many successive capacities.

ARTHUR DENDY, zoologist. He completed for the Challenger Reports the memoir on the monaxonid sponges. At one time Lecturer in Melbourne University and then Professor in Canterbury College, New Zealand, he contributed notable observations on a number of Australasian forms. Work on the pineal eye, the discovery of the ciliated grooves below the brain in the New Zealand lampreys, and a paper on the Reisner fibre of the central nervous system of Vertebrates, are items among his output of work. He was busy with the silicious sponges when his fatal illness overtook him. He had held the Chair of Zoology in King's College, London, for 19 years.

ALBIN HALLER, chemist, foreign member. From pharmaceutical training he had, as is not infrequent in France, passed to a career in pure chemistry. He made an exhaustive study of camphor and its condensation products with aldehydes and ketones. He furnished an important series of papers on the

synthesis of anthracene derivatives. He introduced the idea that the alcohols can act on esters in the presence of hydrogen chloride in the same way that water acts. He was an ardent worker for the cause of technical education, and, largely owing to his efforts, the Chemical Institute was formed at Nancy. For twenty-five years he was Professor of Organic Chemistry at the Sorbonne.

WILLIAM FLETCHER BARRETT had held the Chair of Physics in the Royal College of Science, Dublin, from 1873 to 1910. He made contributions of original value to physics, but devoted his science mainly to the cause of teaching.

FELIX KLEIN, mathematician, foreign member, an eminent geometer and an untiring contributor to almost every branch of mathematics. He will be remembered also as an editor of the "*Annalen der Mathematik*," and as an originator of the *Encyklopädie of Mathematics and Mathematical Physics*, to which he gave long and devoted service.

GEORGE DASHWOOD TAUBMAN GOLDIE, an authority on West African, and especially on Nigerian, questions; he was elected a Fellow under Statute 12 in 1902.

FRANK EVERS BEDDARD, zoologist, and for more than thirty years Prosector to the Zoological Society. His special studies lay in the Oligochæta, and his well-known monograph on that natural order contains some of his best work.

FRANCIS ERNEST JAPP, chemist. His researches dealt especially with certain of the diketones, and belonged mainly to the years when he was Assistant Professor at the College of Science at South Kensington. He later held the Chair of Chemistry at the University of Aberdeen for 24 years.

FRANCIS DARWIN, botanist, whose botanical work included much research, especially into the transpiration and the movements of plants. He was associated with the botanical work of his father, Charles Darwin, and he wrote of him the biography, the well-known "*Life and Letters*," by common consent one of the most admirable and delightful accounts ever written of a great scientific life, the modesty and simplicity of the presentation contributing to its charm. He was awarded the Darwin Medal in 1912. He served as Foreign Secretary of the Society from 1903 to 1907, and was a Vice-President in 1917 and 1918.

EDWIN HENRY BARTON, physicist, Professor in the University College of Nottingham, his birthplace. He was the author of numerous original papers dealing with magnetism, electric waves and with acoustics. He was elected a Fellow in 1916.

WILLIAM SCHLICH, eminent in forestry both as an administrator and as a teacher. Twenty-four years of his earlier career were engaged in India under

the Forests Department of that Government, and there he met continually increasing responsibilities with memorable success. He was then appointed to the staff of the Forestry Branch of the Royal Engineering College at Coopers Hill and subsequently to the Professorship of Forestry at Oxford, from which Chair he retired six years ago. Advanced age slackened but little his enthusiasm and industry, and up to the last he was revising for a new edition his 'Manual of Forestry,' the third revised volume appearing only this year.

JAMES SYKES GAMBLE, another eminent member of the Indian Forest Service. When he first went out to India in 1871, the botany of the Indian forest was for the practical forester's purposes imperfectly known, and the Indian timbers were hardly known at all. Early during his period of service he had furnished by his admirable 'Manual of Indian Timbers' a treatise which has been the standby of generations of Indian forest officers.

ANDREW GRAY, physicist, Professor of Natural Philosophy in the University of Glasgow, his *alma mater*, and where he had, during nine years of his earlier life, been assistant to Sir William Thomson (Lord Kelvin). He was author of a well-known treatise on 'Absolute Measurements in Electricity and Magnetism.'

JOHN YOUNG BUCHANAN, oceanographer. While with the "Challenger" expedition, he demonstrated the inorganic nature of the gelatinous deep-sea deposit which it had been suggested was a primitive living matter. His analysis of certain deep-sea deposits furnished the basis of John Murray's theory of the formation of coral-islands. In his later active life, he collaborated much in the oceanographical work of Prince Albert of Monaco, and in organising the Oceanographical Institute in Paris. Working, however, largely in private laboratories of his own, he accomplished much more than he ever published. His more important papers were issued in a volume from Cambridge, where he resided and was for a time Lecturer on Geography. A member of the scientific staff of the "Challenger" expedition, he had outlived all his fellow-members of it.

JOHN NEWPORT LANGLEY, a physiologist of unswerving reliability and fidelity to research. His earlier work traced the relation between the granules of the living secreting cell and the amount and character of the gland's secretion. In 1889 he, in conjunction with his pupil Dickinson, communicated to this Society the observation that nicotine selectively paralyses the transmission of nervous impulses through the ganglia of the sympathetic system. Using this reaction as a main means he proceeded in collaboration with Dr. (now Sir) Hugh Anderson and others to unravel, in a completeness wholly remarkable,

what had seemed the inextricable maze of the ganglionated nerve paths to viscera and skin. He showed that the principles underlying their anatomical and functional arrangement were of striking and unexpected simplicity. And this, with Gaskell's previous work, put the whole anatomy and physiology of the sympathetic system upon a new basis, opening it up for the further studies of to-day. Incidentally, during that work he showed that the property of double conduction, already recognized for nerve fibre, explains certain spreads of reaction, his axon reflexes. He succeeded Michael Foster in the Chair at Cambridge in 1903. As Editor of the *Journal of Physiology* he rendered long and arduous service. Of the marked renaissance in British physiology brought about in his time it may be said that he himself was one of the forces to whom that renaissance was due. He was awarded a Royal Medal in 1892, and was a Vice-President of the Society in 1904.

Turning now to other events of the year, on July 22nd the Society had the honour and pleasure of receiving a visit from Their Majesties the King and Queen. It afforded an opportunity for showing to the Royal visitors the exhibits prepared for the second annual soirée, and a demonstration on Echo-Sounding for Navigational Purposes given by Mr. F. E. Smith. The Queen graciously added her signature to other Royal signatures in the Society's Charter-Book.

A dutiful address has but a few days since been sent to His Majesty expressing the deep sympathy of the Society in his bereavement by the death of the universally beloved Queen Alexandra.

The generous bequest of £10,000 received last year from an anonymous donor for the promotion of medical research has this year, on the recommendation of the Tropical Diseases Committee of the Society, been resorted to for prosecuting investigation into the disease kala-azar, endemic in India and the Orient. At the instance of the Society, Major Patton and Dr. Hindle started for Northern China in June last in pursuance of that object. And now, as you have seen from the Council's report, the Society has received from the same generous and anonymous source a further munificent bequest of something over £28,000, to be applied on the same terms and under the same condition of anonymity. Those directions will be observed with grateful remembrance.

This year the Society, at the request of the Government, has again organised, through a committee appointed for the purpose, an exhibition of pure science at the British Empire Exhibition. The catalogue of last year's exhibition was revised, enlarged and republished under the title 'Phases of Modern Science.'

It is estimated that the Pure Science Exhibition has been visited this year by at least 120,000 persons. It has thus brought before a wide public the importance of scientific inquiry, pure and applied. Mr. F. E. Smith, the Chairman; Mr. Martin, the Secretary, and their colleagues of the committee, receive our thanks for an arduous effort and our congratulations on the success it has attained.

From the fund accruing to it in 1921 by the bequest of Miss Foulerton, the Society has this year been able, in pursuance of its scheme for advancing Natural Knowledge by the establishment of Research Professorships, to institute a further Foulerton Professorship, and the new Foulerton Professor appointed is Professor A. Vivian Hill. Professor Hill is already universally known as a most distinguished and fruitful investigator in animal physiology. His contributions in that field have been many and of outstanding importance. He has placed the knowledge of muscular contraction—if he will allow me that customary phrase, to which I believe he is purist enough to entertain some objection—upon a new footing. Taking up the problem from the viewpoint which chemical researches had at that time reached, Hill, by his own experiments and experiments in conjunction with his pupils and others, has carried its study much further, especially in its physical aspects. The technique devised and the lines of analysis pursued have been masterly. He has attained preciser measurements, both of the energy changes and of their time relations and of the mechanical work realisable. Examining under various conditions the several ratios existing between these quantities, he has thrown fresh light upon the intimate mechanism of muscle. Not always has it been entirely welcome news that Professor Hill has brought us about our muscles; we learn from him that they are sadly viscous machinery, but to that he reconciles us by pointing out compensatory advantages arising from that property. It is abundantly clear that the more the opportunity he has of prosecuting his inquiries, the more shall we and the world learn of the capacity and intricacies of bodily function. Deeper acquaintance with the principles underlying that function should enable better advantage to be taken of it. Some of Professor Hill's results already touch practical issues of that kind. He is determining decisive factors concerned in the performance and maintenance of physical effort, and is tracing physiological characteristics underlying the skill and endurance of the athlete. Such researches promise information of value in regard to the management of muscular effort and its application on a whole-sale scale to industrial labour. They also promise further insight into what may be termed manual skill. Professor Hill's researches concern, therefore

questions of large practical as well as of theoretical importance. May his tenure of the Foulerton Research Professorship provide him with the amplest opportunity for cultivating the fertile field which has already conspicuously prospered under his able hands.

This Society is one of the oldest of scientific societies, and science grows rapidly, and change is a part of its growth. That the Society vitally participates in that growth and change is evidenced by the increasing stream of papers which pours in upon it. The Society, coexistent as it is in this its third century with many distinguished and flourishing special societies, can view with satisfaction such evidence that it in uncontracted degree supplies a needed channel for scientific issue of new work. The fact testifies that in harmony with its younger sister societies, it provides in unabated measure a living force for the scientific development of its time.

As regards the biological papers brought before the Society, one feature of the time which I think they impress upon the listener, is that at this present the growth of what one may term the experimental biological sciences—physiology, pathology, bacteriology, and pharmacology—is in some measure a convergent growth. Their individual boundaries seem more and more to merge. They are individual in their application rather than in their essential nature, and an advance made by one is of immediate advantage to all. Of any particular paper it would be often difficult, were it desirable, to say under which of these individual sciences it might best be singly classified or catalogued. Nor, under the elastic working of the Society, does that create difficulty; and that again, I think, affords evidence of the practical efficiency of our working arrangement.

With certain stages of growth there goes on the other hand increasing independence of an individual science. This seems so to-day for psychology viewed under the rubric of experimental biology. That psychology is rapidly growing is evident—not least so from its enhanced and successful application to practical problems lying before it in the sphere of industrial management and conditions of labour. Psychology as a part of experimental biology possesses, of course, recognized ties with the physiology and pathology of the nervous system; but on them it no longer explicitly leans to the extent it did. Its discipline becomes more intrinsically its own. This is, to my mind, well, and of favourable augury for its immediate progress as an experimental science. Concurrently with that tendency in psychology it is noteworthy that physiologists, Professor Pavlov and his school, with in this country Dr. Anrep, are pursuing analyses of complex behaviour of the higher animals under

systematic avoidance of all reference, even by implication, to such psychical reactions as accompany that behaviour. Their method applies to animal behaviour, in wider ambit than hitherto, the principles of reflex action. To illustrate by one example:—In his admirable Croonian Lecture in this room last June, Professor Magnus described analyses by himself and his colleagues of the pure reflex behaviour of the cat without cerebral hemispheres. He showed how, for example, a moving mouse before the eyes of such a cat attitudinizes the whole mechanism of the animal, exciting from it appropriate posture and direction in readiness for the final spring upon its prey. After that, “all the cat has to do is to decide to jump.” To jump or not to jump, that becomes the question. At such a point it is that the work of Pavlov and his school dovetails on to the work of Magnus and his school. Pavlov shows how in the intact animal such a final turning-point in its train of reactive behaviour—for instance, “jump” or “not-jump”—can be studied as what is termed a “conditioned reflex”; he shows how that turning-point can be examined as outcome of a balance between physiological impulsions and restraints, dependent partly on conditions under which the act is called for at the moment, partly on conditions under which it has been called for in the past—that is to say, the physiological history of the act in the individual, the mutual time-relations of the dominant stimuli, and so on. The result is thus treated as a sum of physiological factors, positive and negative, interacting under physiological rules, which can be determined, therefore, as obtaining for the cerebral cortex. In this way is pursued a physiological study of higher nervous functions of the animal brain, without appeal to psychical reactions, of which, indeed, the method affirms nothing and denies nothing. To my thinking this line of attack is a gain both for physiology and for psychology, since psychology and physiology, thus treading an essentially common terrain, yet do so each untrammelled by the other and without explicit reference to the eternal psycho-physical problem.

But it would be a far step, and a difficult, and of questionable gain, to carry such divorce of psychology and physiology into the study of fields such, for instance, as human speech, with what that connotes for reaction in the human brain. There it would seem better, as in Dr. Head’s analysis of aphasia, to treat the anatomical, physiological and psychical data together. This seems the better, possibly the only, course of approach to those highest conjoint physiologico-psychological problems, than which there can be few scientific problems which are of greater or more special interest to man.

By reason of the completion of the statutory term of his office, to-day sees

the retirement from the Biological Secretaryship of Sir William Hardy. His tenure included the greater part of the duration of the war, years of unusual difficulty in many ways. Throughout his period of office the Society has owed much to his ability and to his breadth of view. Perhaps not the least among his services have been the catholicity and level width of interest and sympathy he has given to biology throughout its entire range, and even beyond it, and to the field naturalist's side and to the laboratory side alike he has done signal service. I tender him, on behalf of the Society, and in the name of his fellow officers, grateful and hearty thanks.

And let me now—feeling that this meeting will, in accordance with precedent, view my so doing as no pre-election trespass—offer welcome to the distinguished biologist nominated to follow in the office of the Secretaryship. Also let me extend to the name, of unsurpassed eminence in science, nominated for our Presidency a welcome no less hearty. Every good wish to them both.

Before passing to the presentation of the medals, my last official act, let me offer my very hearty thanks to the Society, to the Members of Council with whom I have served, and to my fellow officers throughout the period of my office; also to the Assistant Secretary, Mr. Towle, and to the Assistant Librarian, Mr. White, and to all the members of the staff. My best thanks are due to them, and I tender them sincerely, for the goodwill, kindness and forbearance shown in such full measure to me. The office to which the Society did me the honour of electing me now five years ago I have, I need not say, esteemed as a very great honour; the kindness received on all hands during my endeavour to discharge its responsibilities leaves me its recollection as not only a high privilege but an inefaceable pleasure.

The Copley Medal is awarded to Professor Albert Einstein.

The name of Einstein is known to everyone through the theory of Relativity which he originated in 1905 and extended by a notable generalization in 1915. Previous investigators had discovered that electromagnetic phenomena for a moving system could be correlated to a "local" or fictitious time and space-reckoning, determined by the motion of the system. Einstein realised that the time and space with which we are so directly acquainted by experience can be no other than the fictitious *local* time and space of the moving system—the motion in this case being that of the earth; we have no means of determining, nor can physical science be concerned with, any absolute reckoning of space and time. It followed that the classical scheme of physics, erected on this terrestrial space-time framework, partakes largely of the geocentric stand-

point supposed to have been abandoned from the time of Copernicus. Einstein opened our eyes to the existence of a broader standpoint from which the strange and complicated results of motion—such as the Fitzgerald contraction and the change of mass with velocity—were banished, as the earlier revolt from a geocentric view of nature had banished the complicated cycles and epicycles of Ptolemy. After this Einstein was led to the identification of mass with energy—another result of far-reaching importance, which allows us to know the exact amount of the store of energy so tantalizingly hidden within the atom.

There was a feeling that this theory of relativity for uniform motion must be a particular case of something more general; but observational knowledge seemed to oppose a decisive negative to any extension. It was Einstein again who found the way to the generalization of bringing gravitation into his scheme. Just as the complex phenomena of the moving system had been found to arise in the process of referring its description to an irrelevant frame of space and time, so the phenomenon of gravitation was found to arise in referring a non-Euclidean region of the world to an irrelevant Euclidean frame. At first sight this seems to be an abstract geometrization of a physical subject, substituting an appeal to pure geometry, instead of rational mechanical explanation. But the real effect has been rather to reduce natural geometry to a branch of physics; it is now recognized that the metre rod is a physical appliance for exploring the field of inertia and gravitation, as the magnetic needle explores the electro-magnetic field; and the results of the two kinds of exploration of the world ought not to be given differential treatment.

Einstein's general theory of relativity is remarkable alike for the brilliance of conception and the mastery of the mathematical implement required to develop it. The mathematical technique is now familiar to many, and the new thought has become almost commonplace; but we do not cease to marvel at the genius which first found the way through the overwhelming obstacles. The new law of gravitation must be reckoned the first fundamental advance in the subject since the time of Newton. It involves an interaction between gravitation and light, which had indeed been suspected by Newton and almost taken for granted by Laplace, though it dropped out of scientific speculation when the corpuscular theory of light gave way to the undulatory theory. The three crucial astronomical tests of Einstein's theory have all been verified—the motion of perihelion of Mercury, the deflection of light, and the red-shift of the spectral lines. The last-named proved the most difficult to test, but there is now general agreement that it is present in the solar spectrum. More recently Einstein's theory of gravitation has appealed to astronomers not merely as

something which they are asked to test, but as a direct aid to the advancement of astronomical research. The *tested* has become the *tester*. Invoked to decide the truth of a suspicion of transcendently high density in the "white dwarf" stars, it has decided that in the companion of Sirius matter is compressed to the almost incredible density of a ton to the cubic inch.

The other direction in which modern physical theory has broken away altogether from the ideas of the nineteenth century is in the quantum theory. Whilst there is nothing in the relativity theory too difficult to be mastered with sufficient effort, probably no one would claim that he really understands the quantum theory. For such illumination as we do possess we are in great measure indebted to Prof. Einstein. In 1905, almost at the same time as he published his first work on relativity, he put forward the famous law of the photo-electric effect, according to which the energy of a single quantum is employed in separating an electron from an atom and endowing it with kinetic energy. This was, perhaps, the first recognition that the development of the new quantum mechanics was not to be tied to classical mechanics by pictures of quasi-mechanical oscillators or other intermediate conceptions, but was to proceed independently on radically different principles. Noteworthy contributions followed, on the theory of ionization of material, and on the problem of the specific heats of solids. In 1917 Einstein reached another fundamental result—namely, the general equation connecting absorption and emission coefficients of all kinds. This gives deep insight into the origin of Planck's law of radiation, besides providing new formulæ with the widest practical applications.

If it is thought that Einstein's work of relativity deals with transcendental realms of thought with little bearing on the immediate needs of practical science, his work on the quantum theory has provided some of the most indispensable formulæ of daily application in the physical laboratory. But the truth is that both contribute to the development of physical science in its most practical mood; and both alike are distinguished by a penetrating insight into the origin and interconnection of the laws governing experimental phenomena.

A Royal Medal is awarded to Professor William Henry Perkin.

The science of organic chemistry owes a large debt to Prof. William Henry Perkin, as instance in recent years his monograph on cryptopine and protopine, a record of chemical research rarely equalled in experimental skill and precise reasoning. He has revealed the constitutions of the alkaloids harmine and harmaline; he is nearing the solution of the structures of strychnine and brucine,

two alkaloids which have hitherto resisted all attempts to determine their structural formulæ. His work on berberine has left few questions unanswered concerning the constitution of this important substance. Moreover, while developing new methods of attack on the chemistry of these natural products, he has faced many problems in structural organic chemistry, which, though aside from the main line of his work, he has with characteristic thoroughness solved by the aid of collaborators who have received their training under him.

He succeeded, during a period of twenty years at the University of Manchester, in building up there a school of chemical research which was a pattern to the country, and gave inspiration to many who were fortunate enough to study there under him. During the past twelve years, in the University of Oxford, he has again organized and developed a similar research school; the records of the Dyson-Perrins Laboratory at Oxford, designed and equipped under his direction, bear witness to the extent and fruitfulness of the researches there carried out by him.

For his earlier work the Society, in 1906, awarded him the Davy Medal; this Royal Medal is now awarded him in recognition of his more recent contributions to the science he has done so much to advance.

A Royal Medal is awarded to Professor Albert Charles Seward.

Professor Seward's work has been conspicuous on account of the way in which he has extended and reduced to order our knowledge of the palæobotany of Gondwanaland, especially in India, South and Central Africa, Antarctica and the Falkland Islands. The isolated descriptions of fragmentary fossils, which have accumulated in enormous quantities since McCoy, in Australia, and Feistmantel, in India, did their main descriptive work in the 'seventies, remained of local and limited stratigraphical value until further investigations showed that many fossil fragments previously described under separate generic names belonged to the same species. The important problem of correlating the widely separated Gondwana beds in India and on the three southern continents has been based largely on the plant remains, and has now attained a satisfactory stage on account of Professor Seward's work on material obtained from all the areas concerned. The lower stages of the Gondwana system are characterised by evidences of a glacial climate; and in order more completely to understand the conditions of life that existed, Professor Seward has visited Greenland and otherwise paid special attention to the effect of climate and light in explaining the rise and luxuriance of the *Glossopteris* flora in the Southern Hemisphere. In addition to its direct stratigraphical value to geologists, his work has added

greatly to our knowledge of plant migration, and especially of the way in which the *Glossopteris* flora invaded the Northern Hemisphere, previously occupied by the groups familiar to us by our Coal Measure plants. He has thus utilised the principles and facts of one science to solve the problems of another.

The Davy Medal is awarded to Sir James Irvine.

The constitution of the simpler sugars (monosaccharoses) was based on a sure foundation by the classical researches of Emil Fischer. Taking up the investigation where Fischer had left it, Irvine was able, in the first instance in association with Purdie and later in conjunction with the many students who have received their training in research under him at the University of St. Andrews, to carry the enquiry into the more complex field of the disaccharoses, and by means of new processes, which he has been able to evolve and apply, to assign definite chemical structures to many of these most important natural products. Not content with this achievement, he has also studied the constitutions of the still more complex polysaccharoses, Starch and Inuline, and has been able to throw considerable light on the chemical structures of these substances, incidentally gaining an insight into the manner in which the plant forms and utilises these fundamental reserve materials.

The Sylvester Medal is awarded to Professor Alfred North Whitehead.

Whitehead's early work was strictly mathematical. He made important contributions to generalised algebra, in particular to the calculus of extension, to axiomatic geometry, to non-Euclidean geometry, to the theory of cardinals, and to the older forms of symbolic logic. Always primarily interested in the foundations of mathematics, it is in the logical analysis of these foundations that his main reputation has been won. The great work, *Principia Mathematica*, written in collaboration with Bertrand Russell, contains the most systematic and the most profound analysis to which the foundations of the subject have yet been submitted. In the three volumes of this work it is shown how all pure mathematics may be developed from a minimum of primitive ideas and fundamental propositions, of a purely logical kind, and a standard of accurate reasoning is established, over the whole field of mathematical logic, comparable with that established by Weierstrass in the older mathematical analysis.

From pure mathematics both Whitehead and his collaborator have turned independently to physics. In his more recent books Whitehead has endeavoured to apply the spirit of *Principia Mathematica*, and in particular the principle which he calls "extensive abstraction," in the more complicated and more

controversial field of physical existence. That a point, whether in the older physics or the modern physics of space-time, is a class, or a class of classes, of events, that an electron is a systematic correlation of the characters of all events throughout all nature, are doctrines at which the unsophisticated may be tempted to scoff. The tendency of modern scientific thought is to the conclusion that, if the world of physics is indeed ultimately capable of any rational interpretation, it must be interpreted in some such way. All those, whether mathematicians, philosophers, or physicists, who desire some orderly philosophy of physical nature, will recognise the very great importance of Whitehead's contribution to their common end.

The Hughes' Medal is awarded to Mr. Frank Edward Smith.

Mr. F. E. Smith has been awarded the Hughes' Medal in recognition of the value of his work towards realisation of the fundamental units of electrical measurement. His work on this subject began in 1902.

The results of these various investigations were published in a series of papers in the "Philosophical Transactions," and have remained as standard; such further experiments as have been made since have served only to confirm their accuracy. In words from a paper on the value of an ampere, published in 1912 by the Bureau of Standards of Washington, "The work marks a new epoch in the history of the absolute measurement of electrical quantities."

Other important investigations by Mr. Smith have dealt with the measurement of Terrestrial Magnetism. The recording magnetometers which he designed have proved of great value, while more recently he constructed, at the suggestion of Sir Arthur Schuster, a horizontal force magnetometer of extreme accuracy. During the war his services to the nation were of great importance, and since the Armistice, as Director of Research at the Admiralty, he has been responsible for a number of valuable investigations.

A Critical Statistical Study of Experimental Data on the Effect of Minute Electric Currents on the Growth Rate of the Coleoptile of Barley.

By F. G. GREGORY and L. BATTEN.

(Communicated by Prof. V. H. Blackman, F.R.S.—Received July 15, 1925.)

A study of the effect of very minute electric currents on the rate of growth of the coleoptile of barley was published recently by one of us (F. G. G.) in collaboration.* In this paper the mean rate of a number of control coleoptiles was compared with the mean rate of a number exposed to a minute electric discharge. The growth rate of individual coleoptiles showed, naturally, considerable divergences, so the mean result was in each case based on the observation of a large number of coleoptiles, the increments of growth of individual coleoptiles being stated as percentages of the rate of growth during the first hour of observation. It was assumed that the distribution of growth rates in a comparatively large sample of a pure-line barley would conform with the normal distribution; the probable errors of the mean results were therefore calculated in the ordinary way.

During the continuation of this work positive results have been obtained in further experimental sets, but a number of these, though significant in the mass, were *individually* without significance. This suggested that a careful statistical study of the data on which the results were based might show how the accuracy of the method could be increased. Such a study has accordingly been undertaken, and it seems probable that methods employed are likely to be of use in the treatment of similar data.

The percentage increment basis for estimating the changes in growth rate was employed in the original paper with the idea of rendering more comparable results obtained from slowly and from actively growing coleoptiles. It has the disadvantage, however, that should the growth of the first hour be abnormal, an error is introduced into all subsequent hourly rates, unless all these exhibit the abnormality to the same degree. The method also assumes that the acceleration in growth rate, which all coleoptiles exhibit as they

* V. H. Blackman, A. T. Legg and F. G. Gregory. "The Effect of a Direct Electric Current of a very Low Intensity on the Rate of Growth of the Coleoptile of Barley." 'Roy. Soc. Proc.,' B, vol. 95, pp. 214-228 (1923).

increase in length, is proportional—over the whole range of growth encountered—to the rate of the first experimental hour. A study of the data shows that this is not the case, and that by employing this method the acceleration for coleoptiles with low initial growth rates tends to be too high. These characteristics of the method would not have been of importance if it had been possible to obtain in the various experimental sets samples covering the same range of growth and having the same mean initial rate. An analysis of the data shows that this has not been achieved, and that a few aberrant coleoptiles in a sample are liable markedly to bias the mean result.

As will be seen below a treatment of the data has now been employed which spreads the effect of errors uniformly over the whole period of the experiment. The correlation of the acceleration of growth with the initial rate of growth has also been determined, so that allowance can be made for the behaviour of coleoptiles with different initial rates. Using such adjusted data the results have been recalculated, showing that a case has been made out for the effect of one hour's electrification, at least, the positive result obtained for this short period having a probability of over 200 : 1.

Method of Calculation.

In order to secure uniform distribution of the effect of errors over the whole period of the experiment it is necessary that in the estimation of the effect sought for, all measurements made should have equal weight. This has been achieved by calculating, from the hourly increments in growth, the "straight line of closest fit." The method of least squares was used, and ensured that the mean square deviation from this straight line was the smallest possible. It may be objected that an element of arbitrariness has been introduced by selecting the straight line as the curve of closest fit; but, in fact, straight lines do represent very closely the actual experimental curves over the particular period under consideration. Had this not been the case a curve of higher order would have been used.

Fig. 1 shows the calculated straight line and the mean experimental value for a series of control experiments over a ten-hour period. A rhythm is apparent in the growth rates for each individual plant, and since this rhythm survives the process of taking means, it seems probable that the phase of the periodicity is induced by some condition associated with the setting up of the experiment. The relation between the time taken to complete the cycle and the time interval at which the plants are measured is obviously

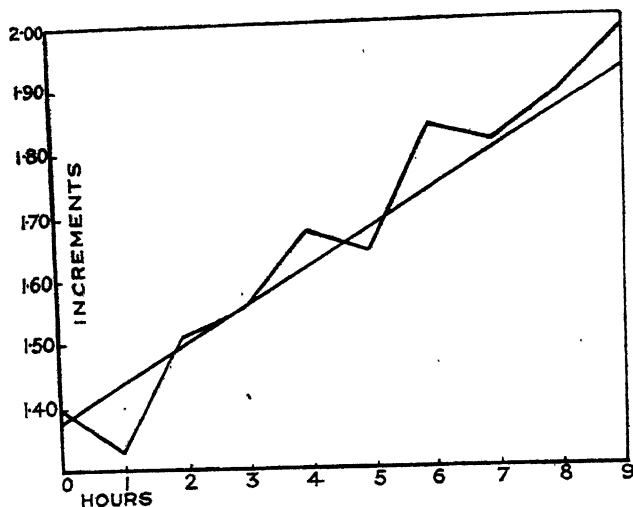


FIG. 1.—Mean values in scale divisions of micrometer of hourly growth-rate of 18 coleoptiles, they exhibit well-marked rhythm. The calculated straight line of closest fit is shown.

important, since it is desirable that measurement should always be taken in the same phase, otherwise a rhythm will appear also in the successive hourly averages, which probably accounts for the observed oscillation.

The constants of the straight line represent two important quantities:—

1. The rate at time, zero, corresponding with the growth rate in the hour previous to electrification.
2. The slope of the line which measures the acceleration in growth.

From the series of growth measurements for each individual, both in the electrified and control experiments, a straight line of closest fit was calculated, and it was these straight lines on which the subsequent calculations were based.

In the general equation for the straight line $y - mx = c$, the slope of the line is given by the value of m , and c represents the point of origin; x is represented in our experiments by the number of hours which have elapsed since the hour previous to the first hour of observation. This was $2\frac{1}{2}$ hours after the experiment was set up and an hour previous to electrification in the case of the experimental plants; c represents the growth rate during that hour and m the average acceleration. From the series of lines thus obtained, the effect of electrification is measured by the difference in the value of mean m for the electrified and control series—i.e., the difference in slope of the lines.

From an examination of all the data two points emerge: (1) The slopes of the lines in corresponding series vary among themselves. (2) Even when

the slopes are similar, the points of origin (the values of c) vary from experiment to experiment, and are different in the control and electrified plants of a single series.

It is to be noted that in each series the value of c for the controls was always above that for the electrified; the use of the percentage method calculation thus always tended to bias the results in favour of electrified plants. This point is clearly brought out in fig. 2, where the plants of a single control series

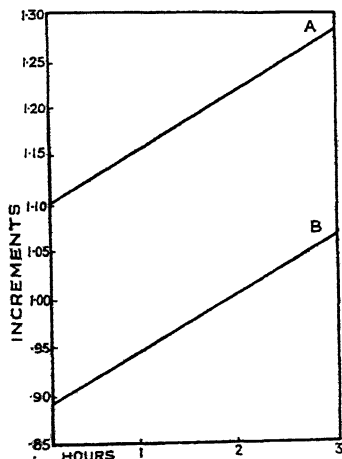


FIG. 2.

FIG. 2.—Straight lines of closest fit for means of hourly increments of growth of a single sample of controls, divided into two sets, A and B, showing the sets with initial growth rate respectively above and below the mean rate of control sample.

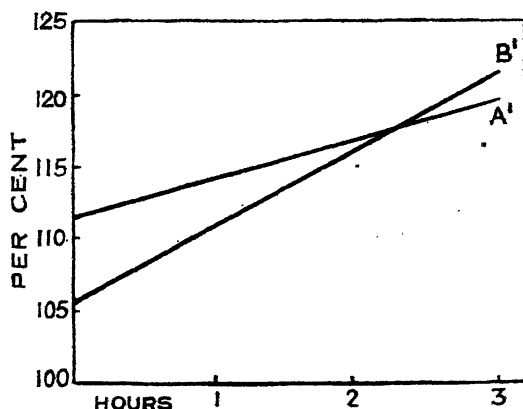


FIG. 3.

FIG. 3.—Straight lines of closest fit for the same two sets as shown in fig. 2, but with increments of growth shown as percentages of initial rate.

were separated into two classes—A and B namely—those with a value of c above and below the mean value for the whole series. The high values correspond with the longer and the low values with the shorter coleoptiles. The data were dealt with in two ways. In one case, the straight lines were fitted to the means of the actual hourly increments (fig. 2); in the other case the straight lines were fitted to the means of the percentage increments (fig. 3). The first method gives two nearly parallel lines having different values of c , corresponding to the longer and shorter average lengths of the coleoptiles. The second method gives entirely different results. The slopes of the lines are now different, and the bias in favour of the shorter coleoptiles is very evident.

In order to compare the electrified and control series, it is obviously necessary to standardise the points of origin of the straight lines, and before this could be done the relation between the slope (i.e., of the acceleration of growth)

and the points of origin (*i.e.*, the initial rate of growth) had to be determined. For this purpose the method of correlation was used, and the correlation coefficients between m and c for each series were calculated. In every case the correlation was negative.

Experiment.		Correlation between Slope of the Line and Initial Growth Rate. $r_{m.c.}$	Regression of Slope on Growth Rate. $b_{m.c.}$
A	One hour electrification. Four hours after effect.....	— 0.295	— 0.0401
	Control	— 0.162	— 0.023
B	Three hours' electrification	— 0.237	— 0.0490
	Control	— 0.0786	— 0.0124
C	After effect of B (two hours).....	— 0.500	— 0.241
	Control	— 0.402	— 0.126
D	Half-hour electrification. Three hours' effect.....	— 0.527	— 0.138
	Control	— 0.416	— 0.330

From the correlation coefficients the regression of slope on growth rate at time zero was obtained, which gave the mean change in slope (the value of m) for a given change in the initial rate of growth (c). The standardised value selected for c in each case was the mean value of c for electrified and control samples, thus enabling a readjustment to be made of the value of m for the differences between the values of c and the standard value. This gave a new series of values for the slopes of the individual lines, (m') corrected for the variation in growth rate during the hour previous to electrification. In this way the effect of heterogeneity in the samples has been corrected.

The following table (p. 127) shows the values obtained for the series of 36 control plants and the series in which a discharge of three hours was given.

The Significance of the Slope of the Line.

The straight lines representing the results of the controls and electrified experiments constitute a sheaf of lines, originating all at the same point and spreading with greater or less slope about the mean lines. The significance of the difference in slope between the mean lines, which is the quantity taken to represent the effect of electrification, will depend on the degree of variation in the slopes of the individual lines. For the estimation of the significance of this difference R. A. Fisher's modification of students' method was used.*

* R. A. Fisher, 'Statistical Methods for Research Workers,' London, 1925.

Controls.			Electrified. Three hours' discharge.		
<i>c</i>	<i>m</i>	<i>m'</i>	<i>c</i>	<i>m</i>	<i>m'</i>
0.988	0.088	0.0880	0.690	0.190	0.1753
1.193	0.088	0.0905	0.890	0.100	0.0951
1.104	0.069	0.0704	0.977	0.157	0.1564
1.139	0.056	0.0578	0.910	0.060	0.0561
1.461	0.036	0.0418	0.759	0.067	0.0557
0.939	0.079	0.0784	1.222	0.082	0.0934
1.421	0.096	0.1014	0.930	0.050	0.0471
1.336	0.086	0.0903	0.815	0.110	0.1014
0.960	0.020	0.0196	1.138	0.058	0.0653
0.683	0.113	0.1092	1.436	0.071	0.0929
0.866	0.126	0.1245	0.958	0.123	0.1214
1.306	0.106	0.1099	0.973	0.068	0.0672
1.045	0.045	0.0457	1.080	0.090	0.0944
0.768	0.068	0.0652	1.044	0.099	0.1016
0.794	0.064	0.0616	0.866	0.091	0.0849
0.894	0.059	0.0578	1.048	0.101	0.1038
0.917	0.092	0.0911	1.166	0.071	0.0796
1.019	0.104	0.1044	0.826	0.076	0.0680
1.287	0.047	0.0507	0.988	0.083	0.0829
0.878	0.098	0.0966	0.861	0.076	0.0697
1.059	0.099	0.0999	0.647	0.077	0.0602
1.131	0.021	0.0228	0.659	0.104	0.0878
0.841	0.061	0.0591	1.002	0.097	0.0976
0.720	0.015	0.0116	0.965	0.005	0.0038
0.823	0.043	0.0409	0.901	0.121	0.1166
0.958	0.073	0.0726	0.988	0.133	0.1329
0.944	0.104	0.1034	0.982	0.092	0.0916
1.070	0.075	0.0760	0.901	0.066	0.0616
1.076	0.066	0.0671			
0.920	0.105	0.1041			
0.980	0.090	0.0899			
1.114	0.089	0.0905			
0.847	0.057	0.0552			
0.952	0.152	0.1515			
0.946	0.081	0.0805			
1.300	0.045	0.0489			
Means— 1.018	0.0754	0.0758	0.951	0.0899	0.0880

$$r_{em} = - 0.07863$$

$$\sigma_c = 0.1899$$

$$\sigma_m = 0.02999$$

$$b_{mc} = 0.01241$$

$$r_{em} = - 0.2374$$

$$\sigma_c = 0.1664$$

$$\sigma_m = 0.03437$$

$$b_{mc} = - 0.04903$$

c initial growth rate.

m slope of line of closest fit calculated for each individual experiment.

m' corrected value of slope, for *c* = 0.990.

The factors entering into the calculation are (1) the mean difference in slope ; (2) the sum of the squares of the deviations from the mean ; (3) the number of individuals examined.

This method has been applied to four sets of data, and the results so recalculated are given below:—

Experiment.	Difference between Mean Values of Acceleration of Growth Rate of Control and of Electrified Plants.	Value of t .	Probability.
A.—One hour electrification. Four hours' after-effect.....	+ 0.01886	2.756	200 : 1
B.—Three hours' electrification	+ 0.0122	1.458	13 : 1
C.—After-effect of B (two hours)	+ 0.00025	0.500	Not significant.
D.—Half-hour electrification. Three hours' after-effect.	+ 0.00393	0.206	Not significant.
	For all results.	2.46	130 : 1

It is to be noted that all the results are positive, and these four electro-culture results taken together are definitely significant of the effect of the discharge, since the sum of the four values of t divided by the square root of the number of sets give a value of 2.46. The individual results, however, vary in significance. It is evident from the value of the acceleration of growth produced by one hour's electrification that the first result is highly significant, the probability being over 200 : 1. Since the positive values alone are considered the probabilities, as read from Fisher's table of t , have been doubled. For three hours' electrification the result is suggestive, with a probability of 13 : 1, but alone is not significant, while the third and fourth results individually lack significance. The possibility of securing a significant result depends largely on the total variance in growth rate of the sample being low, and so far this has not always been achieved. The results A and B indicate clearly how large a part variance plays in determining the significance of results. It will be seen that the mean values of acceleration in growth rate for the two experiments A and B do not differ greatly, and the disparity in their significance is due to the fact that by chance a remarkably uniform series of coleoptiles fell into the first experiment, whereas in the second a small proportion of aberrant individuals was included. Indeed, half the total variance in this series can be accounted for by *two* coleoptiles,* and in the whole four series by 10 per cent. of the coleoptiles. Efforts are being made at present to discover, if possible, some means of recognising such aberrant coleoptiles so that they may be eliminated before the experiment begins.

* If these two individuals in series B are omitted, the results become significant, with a probability of 22 : 1.

As already pointed out, all the four results are positive; in fact, in these and other similar laboratory experiments, *in no single case has a negative effect of electrification been observed*. This suggests that the failure in a number of cases to obtain results *individually* significant is due to the fact that the small effect sought for has often been masked by the variability of the material used.

A very careful selection of the length of the coleoptiles used should do something to reduce the heterogeneity of the material; other factors are also being studied with the view to the further elimination of aberrant coleoptiles. With a reduction in the variance it should be possible to obtain significant results with a very much smaller sample than have hitherto been employed.

Summary.

A critical statistical analysis has been made of the data on which the results described by Blackman, Legg and Gregory, for the effect of electrification on the growth of the coleoptiles of barley are based. It has been shown that the growth rates in even these comparatively large samples of a pure line barley do not show a normal distribution.

The length of the coleoptile at the beginning was naturally found to be correlated with the growth rate, and a negative correlation was established between the initial growth rate and the natural acceleration of the rate which occurs apart from electrification. Any sample containing an undue proportion of coleoptiles with a slow initial rate would show an exaggerated increase of growth rate later, and this would be still further exaggerated by the percentage basis of calculation of the growth increments.

By calculating the straight line of closest fit for the series of hourly growth rates supplied by the data, the growth for each hour was equally weighted in determining the result.

By determining the correlation between initial growth rate and acceleration of growth rate, correction can be made for variation in the subsequent growth rate due to initial rate. Using such corrected data, the growth rates of the control and experimental sets of coleoptiles appear as lines of varying slope.

Such corrected data are calculated for the controls and four electrified sets—three published sets and one unpublished. All of these show *positive* results, and, taken together, provide markedly significant evidence of the physiological effect of the discharge. Considered separately, it is found that the increase in the rate of growth during five hours as a result of one hour's electrification is highly significant, the probability being over 200:1. The effect of a three

hours' electrification is suggestive but not quite significant, giving a probability of 13 : 1; the other two results are individually without significance.

The fact that neither in these four sets nor in any other set has a negative result been obtained suggests that the failure to obtain individually significant results in some of the experiments is due to the masking of the effect by the variability of the material used. In fact in one experimental set two aberrant coleoptiles were responsible for half the variance.

Measurements of the Reversing Spiral in Cotton Hairs.

By W. LAWRENCE BALLS, Sc.D., F.R.S., and H. A. HANCOCK.

(Received August 5, 1925.)

This paper resumes our study of the reversing spiral structure found in the cell wall of cotton hairs,* and presents typical samples of the many thousands of measurements which we have made during the past three years in an attempt to interpret their significance by statistical methods. These measurements were made under the microscope by the use of elliptically polarized light, with eye-piece graticules and mechanical stage, the colour change being checked by direct observation of the structure whenever any doubt existed.

It is not advisable to draw rigid conclusions as to the causation of the spiral reversals from the data here presented, because such conclusions would be pure inference, unsupported by direct observation. We have tried to grow cotton hairs *in vitro*, outside the boll, for such observation, but have failed so far. The data are therefore given as a record of facts, with no more speculation as to their meaning than is needed to make them cohere. We feel sure that their significance transcends the limits of the genus *Gossypium*, and that statistical study of the data, such as is beyond our competence, would give much information about the detailed mechanism of one part of the growth process.

* W. L. B. and H. A. H., "Further Observations on Cell-wall Structure as seen in Cotton Hairs," 'Roy. Soc. Proc.' B, vol. 93 (1922).

Measurements and Symbols.

l = The length of an unbroken spiral is the distance from one reversal of the spiral to the next reversal, without regard to the hand of the spiral.

l_d and l_s = The length of right-hand spiral (dexter) or left-hand spiral (sinister) between two such reversal points.

L = The length of the complete hair cell.

n = The number of separate l units in the complete hair cell.

Hence

$n - 1$ = the number of reversals in the cell, and $\Sigma l_d / \Sigma l_s = nl = L$.

The ratio $\Sigma l_d / \Sigma l_s$ can be determined; it varies greatly from hair to hair, but approaches unity on the average of many.

The values for l range from about 0.01 mm. to 12.7 mm. It will be seen from the curves reproduced in fig. 4 that 0.01 is probably arbitrary, being our class-unit of measurement, and that l may perhaps be imagined as infinitely small in the limit case.

The measurements of l or l_d and l_s can be plotted as frequency distributions for single hairs or groups of hairs, such as are here presented (figs. 2, 3, 4 and 9).

The angle of inclination of the pit spiral in relation to the cell axis has also been measured (fig. 10).

The value L/n is obtained quickly when the frequency distribution is not required, and serves to compare different hairs in respect of their average distance between two reversal points (fig. 5).

In fig. 1 the variation from hair to hair is sketched graphically for 14 hairs, with detailed presentment for one of the 14 to show the variation in a single hair.

Limitations.

(a) We have previously inferred that the reversals might be generated and predetermined during growth in length, though they are not visible until secondary thickening of the wall begins, this process "developing" them like the development of a latent image on a photographic plate. Having considered other possibilities, such as a predetermination reaching back to the pre-extension stage, we see no reason to abandon this inference. But a severe limitation is thereby imposed on us, because, in this respect, the course of the growth history is invisible until it is completed, and has to be inferred from the finished product.

(b) We are as yet unable to watch hairs which are actually growing in length.

(c) An important limitation is placed on interpretation, as distinct from experiment, by the fact that the optical axis is coincident with the fibril axis

at all parts of its reversing spiral. Hence the causation of the reversals can only be physiological and cannot be ascribed to molecular structure.

(d) The differences from hair to hair are enormous (fig. 1).

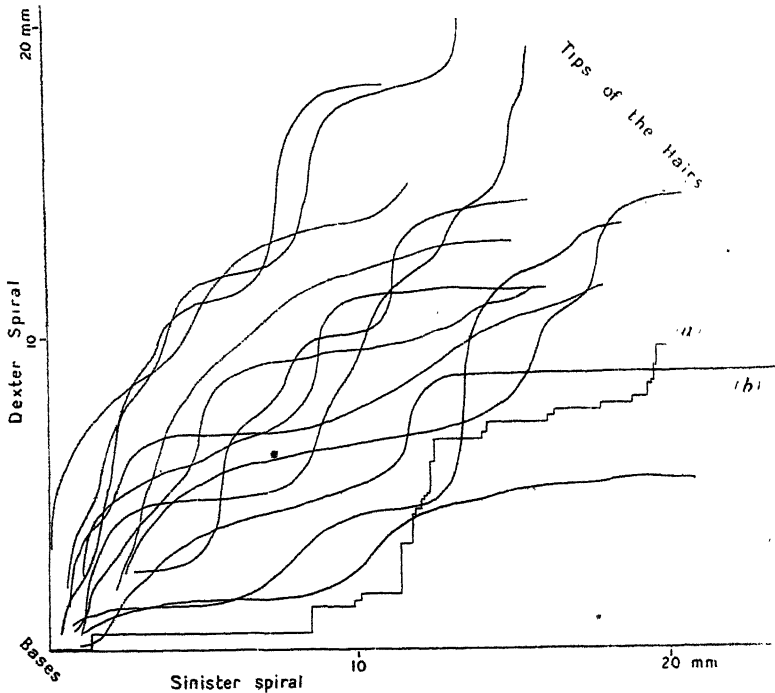


FIG. 1.—Individual Hairs. Uses a convenient method for showing the hand and length of each spiral in any one hair (a) by rectangular plotting. (Compare with the method used in fig. 8.)

By smoothing such graphs the general construction of thirteen other hairs is shown, drawing them with bases coincident. These hairs are taken at random from a fruit which ripened under constant illumination. They include the hair marked (b), which had the longest uninterrupted spiral yet observed, viz., 12.7 mm.

Elimination of Possible Influences.—It will clarify discussion to point out here that we may dismiss various likely causes which might reasonably be expected to have influenced the form of our curves. The elimination of these makes it clear that we are dealing with a phenomenon which is common to all the seed hairs of the genus *Gossypium*, whether those hairs are long lint or short fuzz, and whether they constitute the finest Sea Island cotton or the poorest Indian, or even if they occur on *G. Kirkii*, which otherwise is scarcely recognizable as a cotton plant. It appears, therefore, that we are dealing with a phenomenon of general interest, probably not peculiar to *Gossypium*.

(a) *Genetic Effects*.—Similar frequency distributions of l and nearly identical L/n values were given by Harland's V. 135 pure line of Sea Island, and by Aligarh White Flower Indian, grown side by side (fig. 2). These represent about the best and the worst commercial cottons of the world, approximately *G. peruvianum* and *G. herbaceum*, with 26 and 13 chromosomes* respectively. Similar resemblances were shown by all other cottons examined, the only differences being qualitative and traceable to other causes.

(b) *Environmental Effects*.—We have compared the V. 135 strain and several others (American, Egyptian and Indian), as grown in the field in their native lands, and also as grown in our greenhouse in Bollington under various conditions. The same identity of general type held good (fig. 2).

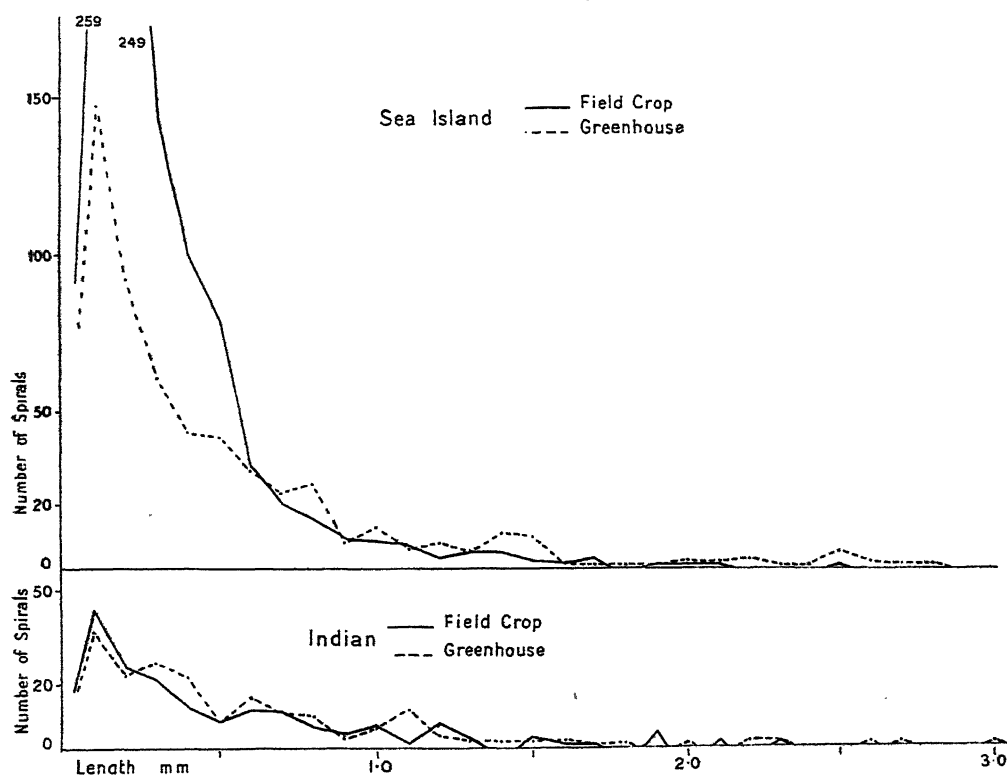


FIG. 2.—Showing the slight effect produced by most extreme genetic and environmental differences upon the frequency distribution of l , the length between reversals. Measured to 0.05 mm.; plotted to class-intervals of 0.1 mm., e.g., 0.95 to 1.00 on ordinate 1.00.

There is no significant difference in form between Sea Island and Indian when grown side by side in the greenhouse, in spite of differences in amplitude. Also, the difference between greenhouse and field for either kind is slight.

* Denham, H. J., "Chromosome Numbers of Old and New World Cottons," 'Shirley Institute Memoirs,' vol. III. No. xxi (1924).

To test our early speculation that the reversals might agree with day and night changes of environment, we grew two plants in constant illumination (5×200 c.p. gas-filled lamps), with only very slight diurnal changes of temperature and humidity. By dis-budding the plants we managed to ripen one boll in 1922 and three in 1923, one of the latter having not only grown in length but having also flowered in the constant environment. We found the usual numbers of reversals, and, again, the usual frequency distribution (fig. 3).

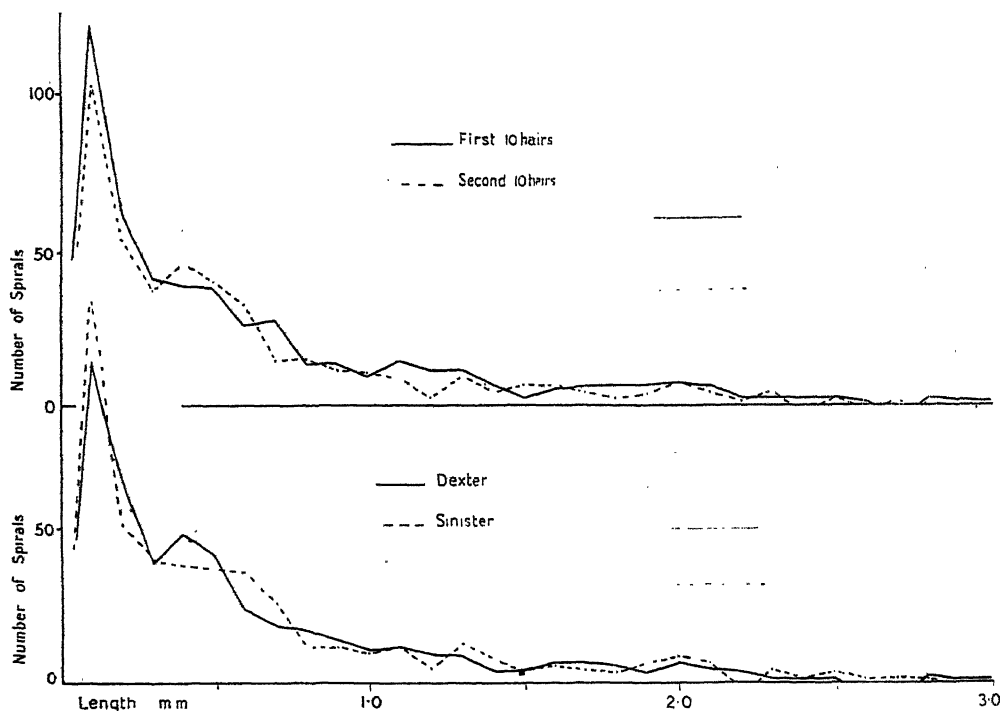


FIG. 3.—Showing that the frequency distribution of l is not modified appreciably by a constant environment. Also, that the difference between the dexter and sinister, l_d and l_s , distributions in long hairs, though slight, is definitely greater than that between the l distributions of equivalent random hairs. Measured and plotted as fig. 2. Material: Egyptian Sakel cotton, transferred from greenhouse to constant illumination; twenty hairs depicted from one boll.

Above:—First ten hairs taken at random. Mean l 0.662 mm.

Additional points, 3.2, 3.5, 3.8, 3.9, 4.4, 12.7.

Second ten hairs taken at random. Mean l 0.657 mm.

Additional points, 3.1, 3.2, 3.3, 3.4, 3.9, 3.9, 4.1, 4.8, 4.8, 6.5, 7.1.

Below:—Dexters only. Mean l_d 0.619 mm.

Additional points, 3.2, 3.2, 3.4, 3.9, 3.9, 4.8.

Sinisters only. Mean l_s 0.698 mm.

Additional points, 3.1, 3.3, 3.5, 3.8, 3.9, 4.1, 4.4, 4.8, 6.5, 7.1, 12.7.

(c) *The Chevelure Pattern*.—Inside a boll the hairs are neatly packed in a sinuous pattern aptly compared to a chevelure. Careful removal of areas from this, without disturbing the relative positions of the constituent hair fragments, showed an entirely random arrangement of the reversals. The ultimate packing of the hairs is thus not a causative factor.

(d) *Location of Seed*.—The following instances from several examinations made on every seed in one fruit will suffice to show the constancy of L/n under such conditions :—

Table I.—Values of L/n in millimetres for One Boll of No. 77 Egyptian, Greenhouse.

Five seeds per loculus and 25 hairs per seed examined for L and n .

	Distal Seed.	Intermediates.			Basal Seed.	Mean by Loculi.
Loculus <i>a</i>	0.56	0.68	0.65	0.63	0.57	0.618
„ <i>b</i>	0.64	0.58	0.60	0.56	0.71	0.617
„ <i>c</i>	0.66	0.58	0.57	0.67	0.63	0.621
Means by locus of seed....	0.62	0.61	0.60	0.62	0.63	

Factors Influencing the Reversals.—A process of elimination next led us to conclude that only two factors were concerned in producing such quantitative variations as we found in our measurements of l . Of these, the chief was the adult length of the cell. If hairs of classified lengths were taken from a single seed the frequency curve was found to become much more skewed in the long hairs, and the L/n value was slightly increased (fig. 4).

The second was less striking in its effects, but far more remarkable in its nature. Using the rate of boll maturation (*i.e.*, extension plus thickening) as a guide to the rate of length-extension of the hair, we found a strong hint of correlation: amongst hairs of the same length the hair which had grown more slowly had longer length intervals between reversals (fig. 5). The factor here seemed to be definitely recognisable as time, which provoked the untenable suggestion that reversals were time-marks, indicating the time-dimension intervals for each cell independently. Later work evaluated this ostensible time factor more reasonably as a matter of chance operation.

Still, however, we had certain abnormalities in the distribution of l , which became more striking as we examined abnormally short hairs. Moreover,

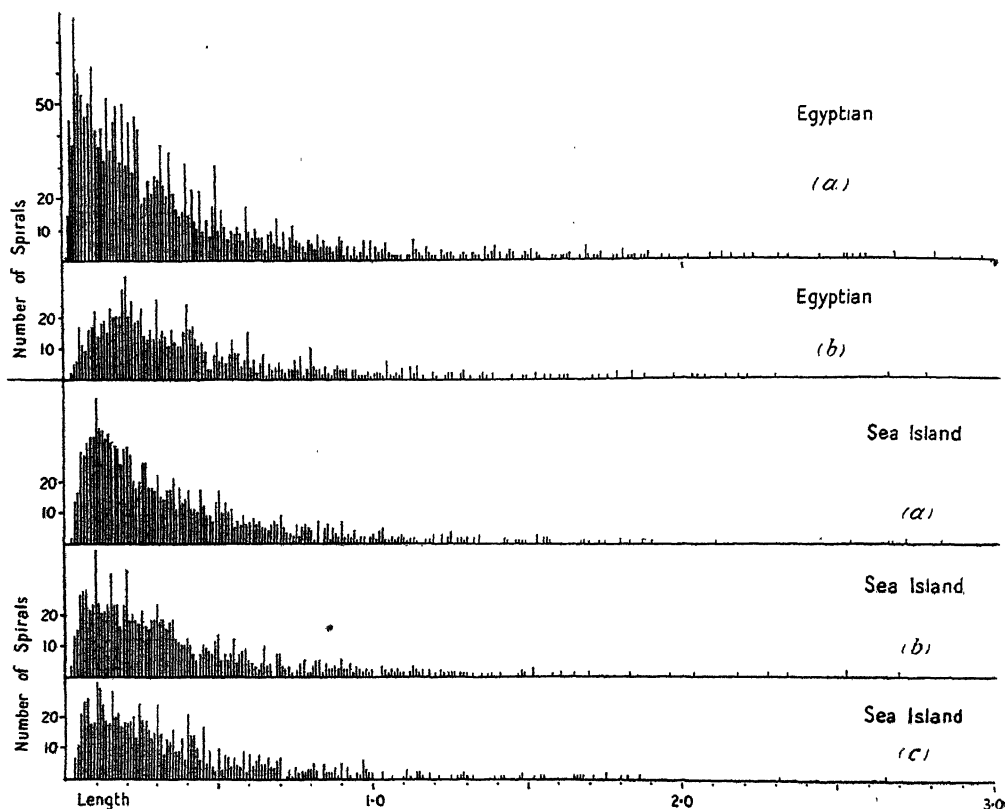


FIG. 4.—Showing the effect of changes in hair-length upon the frequency distribution of l . Measured to 0.01 mm. and plotted to the same, as class-interval. About 6,000 measurements.

Above :—Egyptian pure strain No. 77. One seed of field crop from Sudan.

(a) Twenty hairs of 40 mm. length. $L/n = 0.46$.

(b) Twenty hairs of 20 mm. length. $L/n = 0.44$.

Below :—Sea Island pure strain V. 135. One seed of field crop from St. Vincent.

(a) Ten hairs of 53 mm. length. $L/n = 0.39$.

(b) Ten hairs of 47 mm. length. $L/n = 0.39$.

(c) Ten hairs of 42 mm. length. $L/n = 0.38$.

although our early measurements of l_s and l_d on ordinary lint hairs had shown no obvious differences in these two spirals, we found some dissimilarities in the shorter hairs, and, having recognised them, were able to find them, though less noticeably, in our early data (fig. 3). Evidently the genesis of the dexter and sinister spirals was dissimilar; and so, as our last resort in the method of inference to which we were limited, we turned to re-examination of the fuzz hairs.

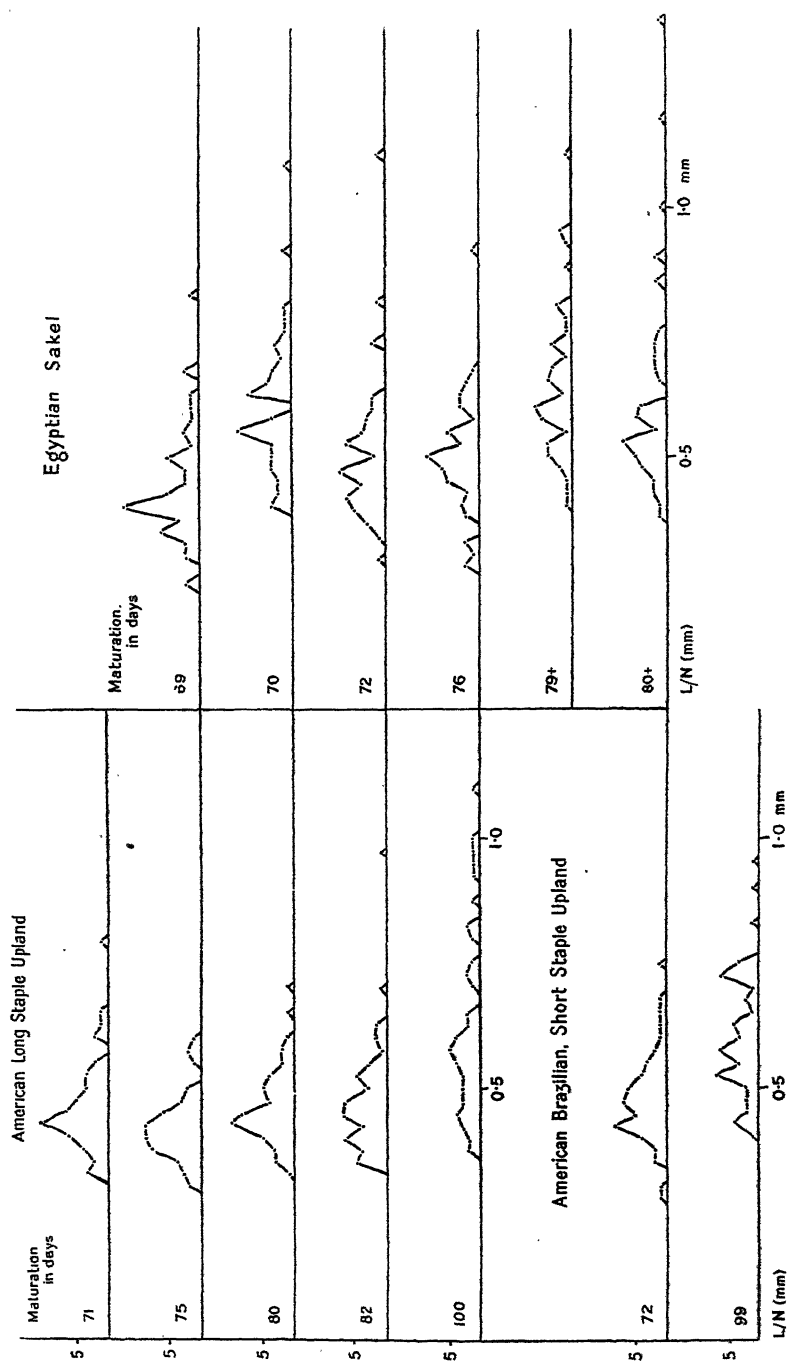


FIG. 5.—Suggesting the existence of a relation between the time during which the hair is growing in length (circa one-half of the maturation period of the fruit) and the average length between successive reversals, or L/n . The value of L/n is plotted for each fruit examined, as a frequency distribution of the individual hairs therein. The material in each of the three kinds comes from one and the same plant, under greenhouse cultivation. The mean values of L/n , with the corresponding maturation periods in brackets, are as follows:—

Long staple:—0.44 (71), 0.41 (75), 0.44 (80), 0.45 (82), 0.56 (100).

Short staple:—0.45 (72), 0.57 (99).

Egyptian:—0.41 (69), 0.57 (70), 0.48 (72), 0.47 (76), 0.60 (79), 0.57 (80).

The Fuzz Hairs.

Previous studies had shown no definite and qualitative distinction between the long lint hairs and the short hairs which compose the velvety "fuzz." We therefore made the working assumption that a short fuzz hair, which may be less than a millimetre long, might—for our present purpose—be regarded as an arrested lint hair; hence, by examining a series of all lengths, fuzz and lint together, we might achieve the same result as if we had been able to take samples of long lint hairs at all stages of length extension, and in their walls develop the "latent image" of the spiral structure. The assumption had evident weaknesses, but since we had ascertained that fuzz hairs sprouted at the same time as lint hairs, and also started secondary thickening at the same time, it was worth a trial.

Growth in Length.—Before considering the series of development stages obtained by regarding fuzz hairs (in respect to the spiral reversals) as samples of arrested normal lint hairs, we may advert to some relevant details.

The growth curve of the hair, plotted as length against time, is sigmoid, beginning exponentially and subsequently brought to rest by a "depressant factor." At all stages the nucleus lies at about two-fifths of the distance from base to tip. The exact mode of growth is unknown, but something can be deduced from the fact that the hair when first sprouting obviously must grow at the tip. This tip growth cannot persist for long, because the final form of the hair is quickly attained at a length of a millimetre or two. For example, if live turgid hairs of various lengths are measured, and the change in cell diameter from base to tip plotted as a "profile diagram" (figs. 6, 7), we find that while adult hairs of different species and varieties have different profiles,* that profile is merely foreshortened in the young hairs. Exact demonstration of this foreshortening is almost impossible, but we have no doubt as to its general truth apart from the initial tip-growth (fig. 6); and from it follows the conclusion that either (a) the whole wall is mobile till length-growth is complete, or (b) that growth is intercalary, the intercalations happening discontinuously and at random in all parts of the hair.

Since the hair may be 3,000 calibres long, and the nucleus position is roughly constant, either supposition seems equally unlikely. We have, however, carefully measured the profiles of some living turgid hairs during secondary

* Henry, Yves, "Détermination de la Valeur Commerciale des Fibres du Coton" (extrait de 'l'Agriculture Pratique des Pays Chauds'), 'Bull. du Jardin Colonial et des Jardins d'Essai des Colonies.'

thickening and find that sudden changes in diameter are quite frequent, which strongly suggest that the tube has been "patched" with slightly over- or

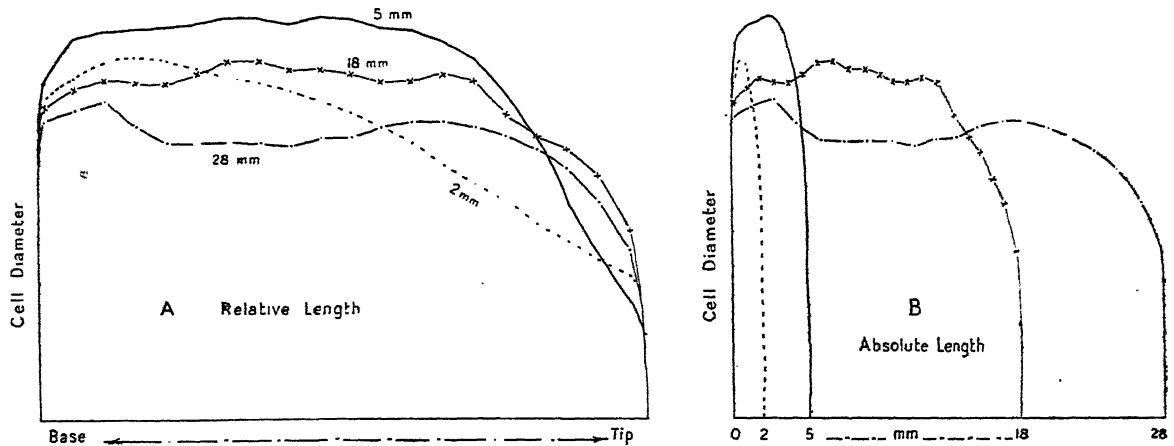


FIG. 6.—Average profiles of hairs of various lengths, showing the approximate constancy of cell diameter at various relative positions along the hair, in all stages of cell-extension excepting the tip.

Exemplified by hairs of various lengths drawn from the same boll of Brazilian American (greenhouse) and measured alive. Mean profiles from four 28-mm., three 18-mm., seven 5-mm. and seven 2-mm. (fuzz) hairs. (Compare with details of one Egyptian hair in fig. 7.)

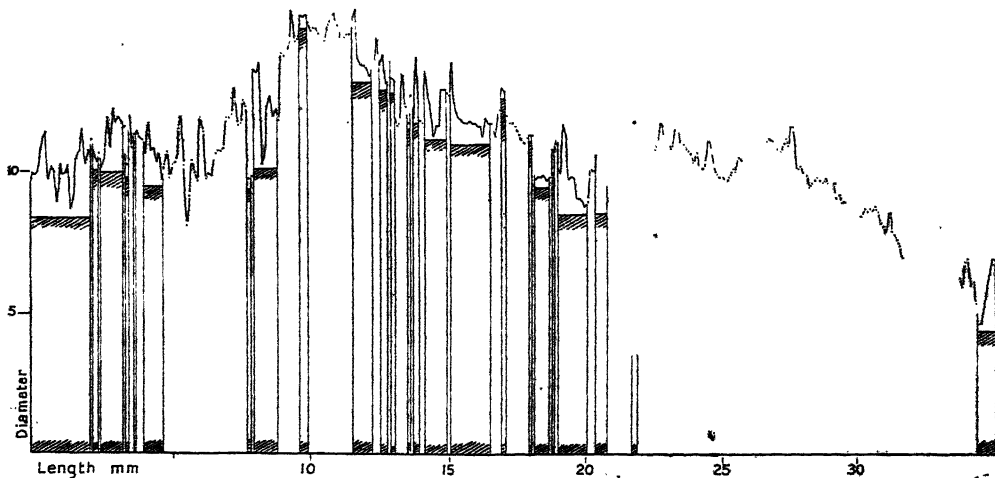


FIG. 7.—Detailed profile of a single living hair, to show the imperfect connection between spiral structure and cell diameter.

Egyptian Affi (greenhouse). In four places the cell diameter could not be measured accurately. Hand of spiral is mapped, the sinister portions being cross-hatched.

under-sized insertions (fig. 7). These occasionally coincide with reversals. We think the balance of evidence, experimental data being unavailable, favours the interpretation that growth takes place by random intercalation. Occasional deformities, such as branches, dog-legs, whip-like tips, etc., indicate that no very rigid procedure is followed by the growing hair, and give further support to this view.

The Predominant Basal Spiral.—We now turn to the fuzz-lint series. The mature fuzz hairs are readily examined, e.g., on sections of the seed coat, in such a way that their undamaged base can be clearly seen. We find that about 94 per cent. of the fuzz hair bases have the same sinistral spiral. The exceptions seem to be real; but these hairs with dextral bases show no other points of difference, and it might be that they also are sinistral for the first few microns only. In any case, nearly all the fuzz hairs, if not quite all, would seem to start growth by making a sinistral spiral (fig. 8).

We have been at pains to attempt similar observations on the lint hairs; but the uncertainty attaching to observations on the distorted bases thereof is too great. In a very limited number of fairly dependable hairs we were satisfied that almost all had again the same sinistral basal spiral.

Moreover, not only have the great majority of lint and fuzz hairs the same basal spiral, but this is sinistral in all the cottons we have examined, whether Sea Island, Indian, Egyptian or American. This difference in the genesis of the l_s and l_d frequency distribution fully accounts for the difference in their forms and for the fading of that difference as the hair grows longer (fig. 9).

The Tip Spiral.—In contrast to the basal spiral, the tip spiral may be of either hand. However, if attention be confined to the fuzz from a single seed, differences from another seed in the same boll may be detected. Thus, in two such seeds, one had a large excess of sinistral tips, the other of dextral tips (figs. 8, A and B). We interpret this to mean that the unknown cause of reversal took effect on most of the population in the latter case while tip growth was still going on, but was delayed in the other until tip growth had ceased, and the reversed dextral spiral was consequently intercalated behind the tip, this itself remaining sinistral, being merely a fragment detached from the base.

The Building of the Frequency Distribution.—We are now in a position to infer the process by which our frequency curves of l_s and l_d and l are built up. For simplicity's sake, we will disregard the 6 per cent. of hairs which start with a dextral spiral base, merely noting that its presence blurs the sharpness of the results. We shall also restrict ourselves to groups of hairs with the same adult length (fig. 9).

When the hairs sprout an l_s distribution is formed, momentarily symmetrical, but becoming skewed by the first hair to show a dextral reversal; because such reversal arrests the elongation of that particular l_s unit, and further lengthening of that hair is confined for a time to the dextral spiral. Thus we start to build also an l_d distribution, whose form is generally similar to that of the l_s one, but which, having started later, must always lag behind the latter in subsequent changes of its form.

The change in form which both these distributions must equally undergo, once tip growth has ceased, will evidently arise from three causes:—

(a) Increasing length of the l units by growth without reversal. This will tend merely to broaden the spread of the curve. Whether it makes for a symmetrical curve or a skewed one is not quite clear. The answer would throw light on the growth mechanism; we think it would be skewed.

(b) Fragmentation of the l units by insertion of a reversal. First the basal l is fractured into two halves, usually unequal, by an inserted l_d . Then, at the next shift of the growing region, another fracture takes place, in any part of the hair, l_d or l_s . We think the evidence indicates that growth alternates regularly between dexter and sinister; but the elucidation of this from our data requires greater statistical skill than we possess. Even if we do not assume this, our third cause still follows.

(c) Addition of l_d to l_d will happen whenever dextral growth fractures a dextral spiral, and, conversely, for l_s to l_s . There will be no structural indication of such an episode, but it is significant that the sudden changes of cell diameter, which have been already mentioned (fig. 7), are at least as common in the middle of an l_d or l_s as they are at the junction between l_d and l_s . Unfortunately, statistical study of this point seems impracticable.

Thus the hypothetically symmetrical initial distribution is progressively altered. Increased length, first of l_s and then of l_d , simply represents greater likelihood of fracture. Each fracture of any one initial l shifts the mode nearer to zero, and the newly intercalated portion similarly undergoes the same exposure to chance. Consequently, in very long hairs, the mode is closer to zero, and yet the long reversals are rather more common than in the shorter hairs. There seems to be a general tendency to approximate to $n^2 = K$, beyond half a millimetre.

There are no consistent differences between different parts of the adult hair in their l distribution, except a tendency to accumulate shorter ones at the tip and the base of the hair. Different samples have shown various characteristic distributions when the hair is divided, *e.g.*, into tenths; but

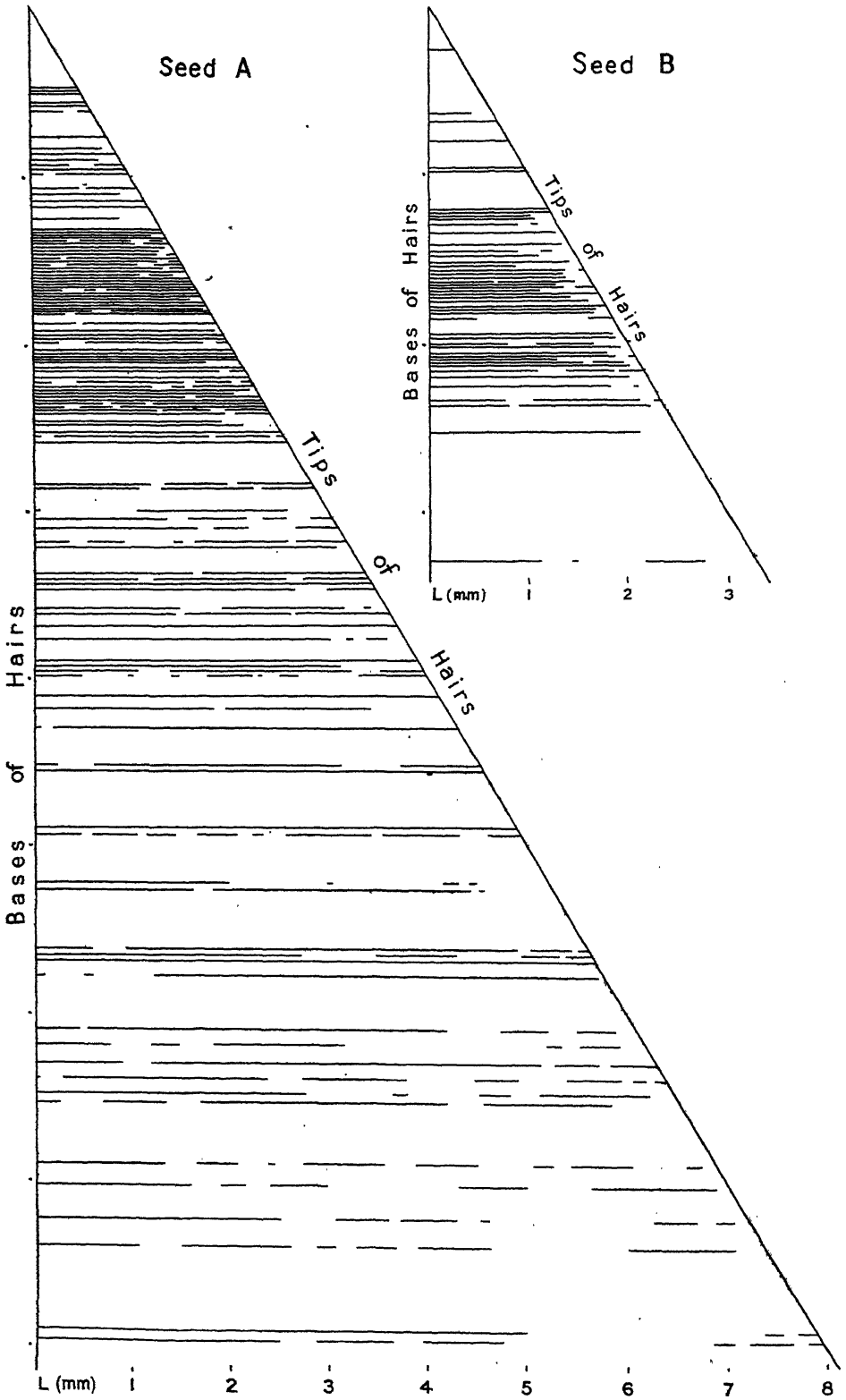


Fig. 8 (see description on page facing).

FIG. 8.—Showing the structure of individual fuzz hairs, which are arrayed in order of their length. The frequency distributions of l_s and l for these hairs are plotted in fig. 9. Sinister spirals are here shown by black lines, the dexter spirals being left blank.

Dexter basal spirals are omitted, viz. :—

Seed A.—7 basal dexter in 123 hairs, or 6 per cent.

Seed B.—3 basal dexter in 50 hairs, or 6 per cent.

Comparing the two seeds for lengths under 3.3 mm., the number of dexter tip spirals is :—

Seed A.—21 dexter tips in 85 hairs, or 25 per cent.

Seed B.—35 dexter tips in 50 hairs, or 70 per cent.

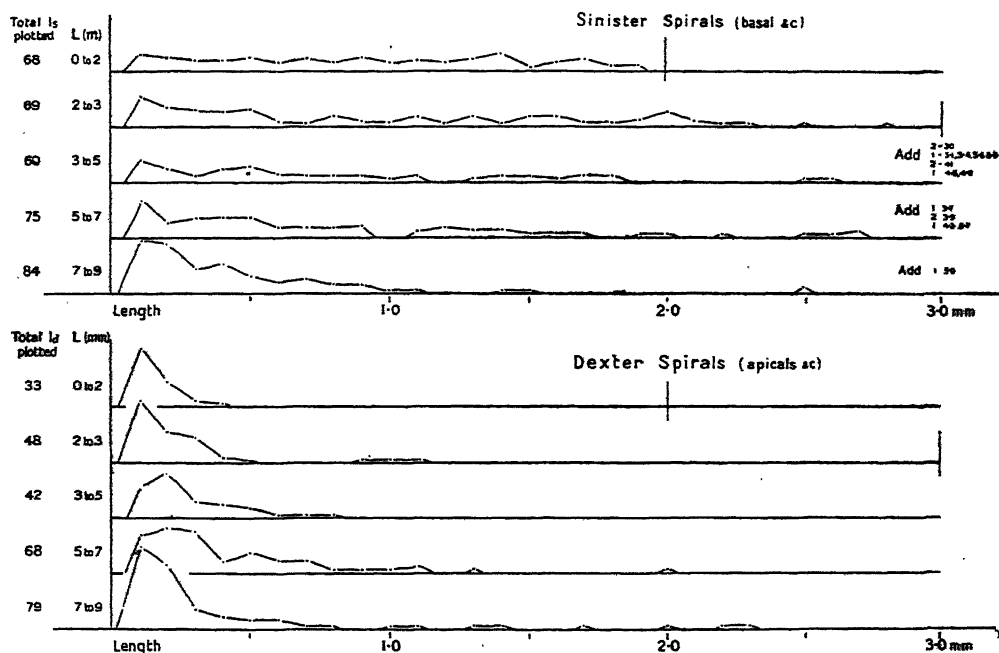


FIG. 9.—Frequency distributions in fuzz hairs of various lengths, to show the growth and fracture of l_s and l . Sinister spirals reach a temporary maximum in 3.5 mm. hairs. Dexter spirals show a primary mode which moves to the right by growth, while a secondary mode is formed by fracture to the left of it in the 5.9 mm. hairs. Scales and plotting are the same as in figs. 2, 3 and 4. The actual hairs are shown in Fig. 8A.

the variations, though recognisable, are not striking enough to give evidence in the absence of actual growth records.

Constancy of Spiral Angle at Reversal.

Perhaps the most strange peculiarity of these reversing spirals is seen when the pit spiral angle is measured (as projected in the horizontal plane at

the cell axis) and plotted to show its variation along the hair. It varies quite appreciably around a modal value, the correlation of this with cell diameter being about 0.2 only; but these variations are smoothly sequential along the hair—and this, in spite of the complete reversal, say, from 30° dexter to 30° sinister, which may happen anywhere (fig. 10).

We have explored the possibility that these angular variations might be post-extension artifacts, but cannot find anything more than small correlations with cell diameter and wall thickness; so it would seem that they are largely original. Moreover, since stress in torsion would be the most likely cause of such variation, we should expect the angles on either side of a reversal to have any initial difference exaggerated, not decreased, by such stresses.

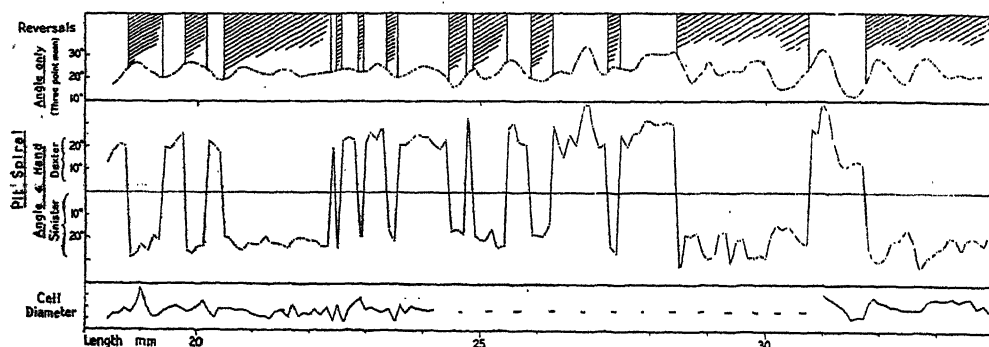


FIG. 10.—The Spiral Angle.—Showing changes in hand and angle of the pit spiral along 15 mm. of one hair. The changes of the angle are independent of the reversals, and are only slightly connected with the changes in cell diameter; from 19.4 to 24.2 mm. the correlation coefficient for angle and diameter is 0.18. In the plotting of "angle and hand" the bimodal grouping round 27° dexter or sinister is evident.

The clue to the whole history of the spiral structure seems to reside in this reversal of hand without change of angle; but we cannot interpret it without speculation, which, in the absence of direct observation on growing hairs, is mere hypothesis.

The following observation, at present also inexplicable, will have to be fitted into the story eventually:—

Collapse Resistance of Dexter and Sinister Spirals.—We have insisted on the fundamental importance, in any interpretation, of the fact that the optical axis follows the fibril axis, thus indicating that the spiral can only have been generated by either (a) building along a spiral path; or (b) by torsional displacement. An observation that the l_2 and l_3 units had different structural

properties in certain hairs seems to hint at the existence of difference in physiological history, even if it does not imply difference in molecular structure.

While re-examining some hairs which had been previously measured for their diameter while alive, and had then died in normal saline solution, we noticed that some portions had collapsed while others remained cylindrical; the sturdy tubular portions were always dextral, the collapsed portions were of the same hand as the basal spiral, *i.e.*, sinistral. Not many hairs have yet shown this phenomenon; but, such as do, have all been strong in the dexter parts and weak in the sinister (fig. 11).

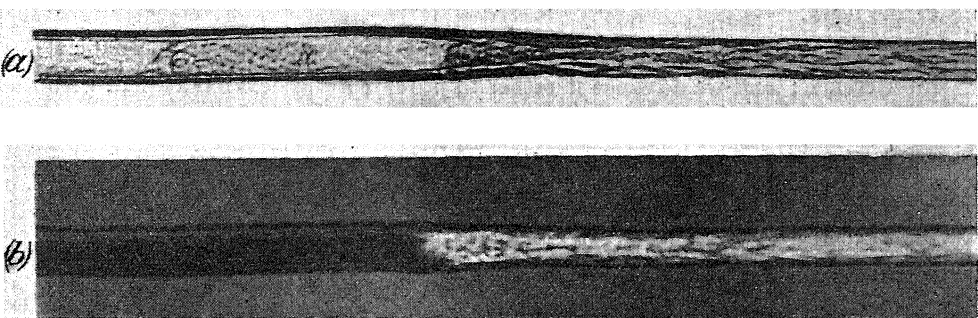


FIG. 11.—Showing sinistral spiral collapsed while dextral remains tubular. Same hair viewed (a) ordinary light, (b) elliptically polarised light.

A Possible and Simple Cause of the Observed Phenomena.

We are dealing with individuals in a population, each individual having its own growth history. If we are right in regarding the spiral reversals as records of that history, no two hairs over 1 mm. long which we have yet examined have had the same history (*e.g.*, fig. 1). Nutritional variation does not seem adequate as an explanation, since hairs growing in proximity to one another are in no way more unlike than those at opposite sides of the seed, nor is there any resemblance between the two arms of a branched hair. To take the elementary case of the first dexter reversal from the basal sinistral spiral, there must be a cause at work, since one seed may effect this reversal sooner than another; but, even so, there seems to be a large residuum of purely chance effect.

The statistical results we have noted 'could be produced if there were mechanical interference of one hair with another. Assume that each cell-tip rotates as it elongates, owing to spiral growth; assume also that contact with

a neighbour, hindering that rotation, serves either as an obstruction from which the cell can only free itself by reversing its rotation, or as a contact stimulus producing a similar effect. The chances of such mutual interference in such a writhing mass would be entirely random.*

This suggestion implies relationship to growth processes elsewhere. Thus, the postulated rotation of that part of the hair which is distal to the temporary growing region would seem to have some resemblance to nutation in multicellular organisms. Again, the postulate of spiral growth into a helix resembles the conception of a fundamental spiral in phyllotaxis, such minor fluctuations as we find in the pit spiral angle being due to the third dimension (length) of the helix.

We are most diffident as to the validity of this hypothesis of mechanical interference; and we advance it rather to provide a physical model than with any conviction of its truth.

The Geometry of the Reversal.—In the early stages of our work we tested out a hypothesis that dexter and sinister were formed simultaneously, like a chevron; but this chevron-hypothesis will not fit the facts.

The insertion of either hand of spiral into pre-existent wall might be due to a "focussing" of anabolic activity at one growth-centre if this growth-centre moved round the inner circumference of the cylindrical wall. Two possibilities then arise:—

(a) It might rotate always in the same direction, counter-clockwise, but could build forward (distally) or backward (basally). This would produce a sinistral spiral in the forward direction, and conversely.

(b) It might build always towards one end of the hair, *e.g.*, distally, but the direction of rotation of the growth-centre would change to clockwise when a dextral spiral was being pre-determined.

Of these the first is the more likely alternative. It only requires local displacement of the hair, and makes the hypothesis suggested in the previous section mechanically practicable. The piecing up of fibril to fibril presents no difficulties in either case, on the analogy of crystallization, new micellæ or unit-cellulose-aggregates being joined end to end at their slip-spiral-surfaces.

The Salient Peculiarity of the Data.—We think our inferences may be trusted to the extent of regarding rotation in growth as a reality. If so, we are then confronted with the fundamental problem which these hair cells have to offer, namely, why have they no stable growth except by rotation? An explanation

* An average boll of Egyptian cotton contains over 100,000 hairs, each 3 cm. long, in a space about 5 c.c. in volume.

based on molecular structure has only one shred of evidence to support it (fig. 11) and without appealing to that structure a physiological interpretation would have little meaning. Experiments on the growing hair should eventually eliminate some of the other present possibilities ; and meanwhile we would only note that one of these transcends the limits of three-dimensional geometry.

Summary.

(a) The paper embodies selections from a mass of statistical data describing the dimensions and form of the spiral arrangements which occur in the cell wall of cotton hairs.

(b) The spirals may be dexter or sinister, and their reversals are apparently predetermined during growth in length. Genetic and ordinary environmental influences do not affect the statistical peculiarities of the reversals.

(c) The final adult length of the hair, and the time taken in reaching that length, do affect the reversal distribution.

(d) Nearly all the seed hairs of *Gossypium* begin to grow on a sinistral spiral, i.e., the opposite hand to an ordinary screw thread.

(e) This basal sinistral spiral increases in length, is broken up, and later additions may be made to its fragments. Similar extension, fragmentation and subsequent addition take place with the later dextral spiral.

(f) The angle of the helix varies somewhat around two modal values, viz. approximately 27° dexter and 27° sinister.

(g) The local variations of the angular value are quite unaffected by inversion of the " hand " of the angle from dexter to sinister.

(h) Dexter and sinister wall structures have been found in some hairs to have different structural properties in their resistance to collapse after the death of the cell.

(j) A tentative explanation of the causation of reversal is suggested ; but attention is directed to its insufficiency and to the need for experimental evidence.

We are indebted to the Fine-Cotton Spinners and Doublers Association for the resources of the greenhouse and microscope equipment with which this work has been done by us in their Experimental Department at Bollington.

A Spirometer Method of Studying Continuously the Gaseous Metabolism of Man during and after Exercise.

By K. FURUSAWA.

(Communicated by Prof. A. V. Hill, F.R.S.—Received September 21, 1925.)

(From the Department of Physiology, University College, London.)

The instruments generally employed for the investigation of the gaseous metabolism in man are divided into two classes, the closed and open circuit systems. Apparatus belonging to the former class is naturally not suitable for a study of rapidly altering gaseous exchange; apparatus of the latter class involves the use either of a spirometer or of a bag for collecting the expired gases. A method employing a series of Douglas bags was devised by Campbell, Douglas and Hobson (1) to follow changes in the gaseous metabolism, and recently this method has been developed in a convenient way by A. V. Hill, Long, and Lupton (2). During rapid ventilation of the lungs the latter authors succeeded in obtaining reliable results by allowing as small an interval as 10 seconds for the collection of a sample of the expired air, during the early period of recovery from severe exercise. In the present paper a method is described which obviates the necessity for a series of bags, and allows a continuous determination to be made of the gaseous metabolism in man, during or after any kind of activity in which the subject does not move away from the apparatus.

Principle of Method.

In fig. 1 curve V denotes the total volume of the expired gas up to time t . Suppose that a sample be taken at time t_n , and that the total volume of the expired gas up to that moment is V_n . From the analysis of the sample and from the total volume of the expired gas we may calculate the amounts of oxygen used and of CO_2 expired by the subject during the time interval t_0-t_n . By increasing the number of samples taken we may make a continuous curve, if we please, of either (a) the percentage of oxygen in the total expired air up to any moment t ; (b) the percentage of CO_2 in the total expired air; (c) the total volume of oxygen used up to time t ; or (d) the total CO_2 expired up to the same time. In fig. 1 the curve denoted by V represents the total volume of oxygen used. From the latter curve the oxygen consumption per unit of time at any given moment t_n is obviously given by the slope of the tangent to the curve at that time. The oxygen used in any finite interval is immediately obtained by

subtracting the ordinates at the beginning and end of that interval. In fig. 1 the broken line represents the rate of oxygen intake obtained by measuring the slope of the curve V . In the examples given below, however, for the sake of

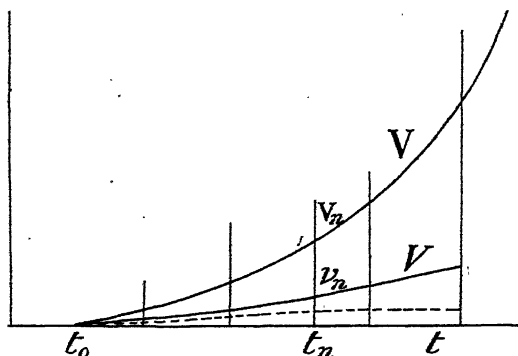


FIG. 1.—Abcissa = time. Ordinate: curve V , total volume of expired gas; curve v , total volume of oxygen taken in; broken curve rate of oxygen intake.

simplicity, finite intervals are employed, and the mean value of the rate of oxygen intake calculated by dividing the total oxygen used during the interval by its duration.

Description of Apparatus.

A spirometer of large capacity is employed. Fig. 2 is a photograph of the

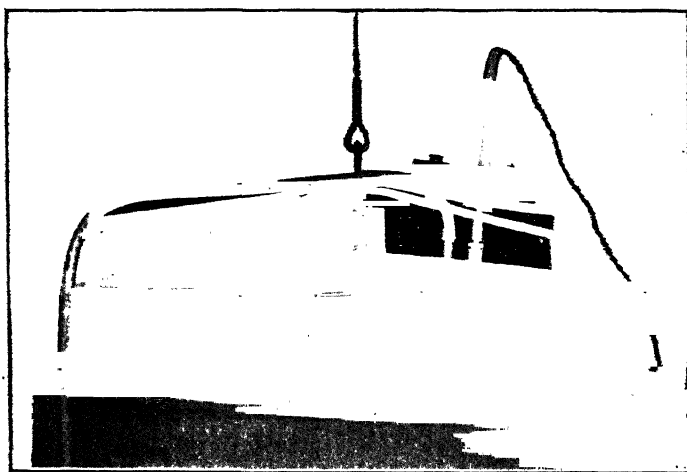


FIG. 2.—Photograph of spirometer, showing inner and outer cylinders, fan motor, cap, sampling pipe, supporting frame and flexible wire.

top of the apparatus, showing the outer and the inner cylinders. A vertical section through the middle line is shown in fig. 3.

The outer cylinder, standing on an iron base 20 cms. high, is of sheet iron galvanized on both sides. Its dimensions are: diameter, 85 cms.; height, 158 cms.; capacity, 900 litres. It is filled with water up to 3 cms. from its upper edge. A hole is bored in the centre of the bottom, through which an iron pipe T for the expired gas enters the cylinder.

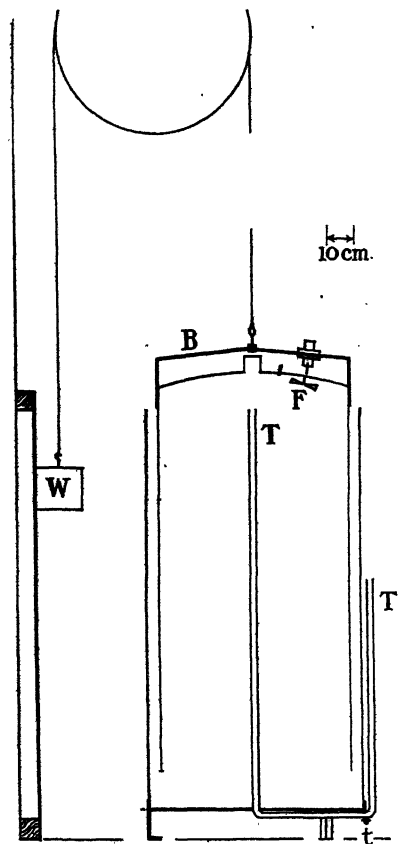


Fig. 3.—Diagram of spirometer. B, steel frame; W, counterweight; T, iron inlet pipe; F, fan; t, drain cock for T.

The inner cylinder is of sheet copper and weighs about 25 kgs. Its dimensions are: diameter, 77 cms.; height, 155 cms.; capacity, 700 litres. Its upper surface is dome-shaped with a slope at the edge of 20°. A small copper cap, 6 cms. high and 7.5 cms. in diameter, is fixed round a hole in the centre of the dome. This cap passes over the steel inlet pipe T and ensures that the dead space is small. The inner cylinder is supported by two steel bars B (one only shown in fig. 3), screwed to its side and crossing above its top. From this steel frame a flexible steel wire runs upwards, passes over a bicycle wheel fixed on the wall of the room near the ceiling, and is then connected to the counterweight W. W is about 0.7 kg. lighter than the inner cylinder. The pressure exerted by this difference of weight on the inner surface

of the copper cylinder is only about 0.4 gm. per sq. cm.

The expired gas enters from the galvanized iron pipe T, which is connected at its outside end by a corrugated rubber tube to the mouthpiece. In order to allow for very rapid ventilation a tube of 2.5 cms. internal diameter was chosen. The top of the iron pipe is just inside the copper cap when the whole of the gas has been pushed out of the apparatus. The total dead space is about 1.7 litres, of which nearly all is in the pipe T. A small tap t is attached to the bottom of

the large pipe to let out water which has passed, either accidentally or by condensation, into the inside of the pipe.

An essential feature of the whole apparatus is the maintenance of a constant composition of the gas throughout the inner cylinder. For this purpose a small fan *F* is fixed inside at the top of the copper cylinder. This is driven by a shaft running through an air-tight gland, by means of a motor placed above on one of the arms of the iron-supporting frame. A short length of rubber tube is used to connect the shaft of the motor to the shaft of the fan. By this means the necessity of extreme accuracy in alignment is avoided and friction between the shaft of the fan and the gland is kept as low as possible. This fan is run continuously during an experiment to keep the air inside the cylinder completely mixed.

A small tube is let into the top of the dome to enable samples to be withdrawn.

Sample Taking and Volume Recording.

To the short brass tube let into the top of the cylinder is fixed a thick rubber tube, 2 mms. in internal diameter and 2.5 metres long, through which samples of the expired gas are taken. A usual type of sampling tube is employed, together with an evacuated glass bottle of about 200 c.c., which is used to clear all gases from the dead space of the rubber tube (about 20 c.c.) before the sample proper is taken. This bottle is evacuated and connected to a side tube of the sampling tube. By turning the tap of the sampling tube, the gas inside the rubber pipe is drawn into the bottle and then, by a further turn of the tap, a pure sample of the gas in the cylinder is taken into the sampling tube.

The volume of the expired gas is simply measured. A piece of board 18 cm. by 90 cm. is fixed on the wall, and along the board slides the counter-weight, which is used as an indicator of the volume. This board is marked in 10-litre divisions. For recording the volume of the expired gas as a function of time, and also the moment of taking a sample, a simple electrical arrangement is employed. Each time that the upper edge of the indicator weight, *W*, passes a 10 litres mark, and also at the moment when a sample is taken, the observer gives a signal: these signals are recorded on a revolving drum, and from the record a chart is afterwards constructed of the type shown in curve *V* of fig. 1.

Examples.

1. *Basal value.*—As was obvious, if the method be properly applied, the results agree with those determined by the Douglas bag technique. Experiments were made by both methods and the agreement was excellent.

2. *Recovery phase.*—The spirometer was originally intended for use in the study of the later period of the recovery after exercise, when the ventilation was small. Actually it is capable of following the whole process of recovery. The two examples shown in fig. 4 illustrate the use of the apparatus in following the recovery phase after muscular exertion.

The two sets of points shown in fig. 4 agree closely with one another, and the resulting curve is exactly similar to the curves given by Hill, Long, and Lupton

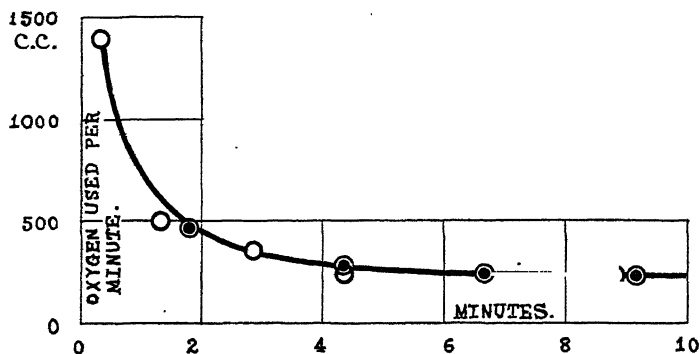


FIG. 4.—Two experiments on recovery of subject A.C. "standing-running" for two minutes, speed 142 steps per minute.

(2) and by Hill and Lupton (3). The method is obviously usable even when the ventilation is heavy. It has, moreover, one valuable property in studying the recovery process in man, namely, that the continuous record of the lung ventilation indicates approximately how the recovery is proceeding. In this sense it acts as though we put a gas meter of low resistance in the air circuit.

3. *The initial phase of exercise.*—The oxygen intake from the beginning of exercise was studied by Hill and Lupton (3), and they found that in running the oxygen intake rises rapidly when the exercise is started, and attains a constant level, depending upon the severity of the exertion, some time after the second minute. This phenomenon also has been studied by the method described here for the case of "standing-running." The curves of fig. 5 show some of the observations made on several different individuals:—Subject B.: speed, 140 steps per minute. Subject M.K.: speed, 180 steps per minute. Subject J.: speed, 180 steps per minute. Subject C.: speed, 270 steps per minute. The lowest curve, representing very moderate exercise, reaches a constant level after one minute. At the intermediate speed, represented by the middle curve, two subjects of different nationalities gave identical results. This curve also shows a constant level higher than the first mentioned, which is attained during the

second minute. The highest curve was observed on a subject of good physique, moving at a speed which it was impossible for him to maintain for more than

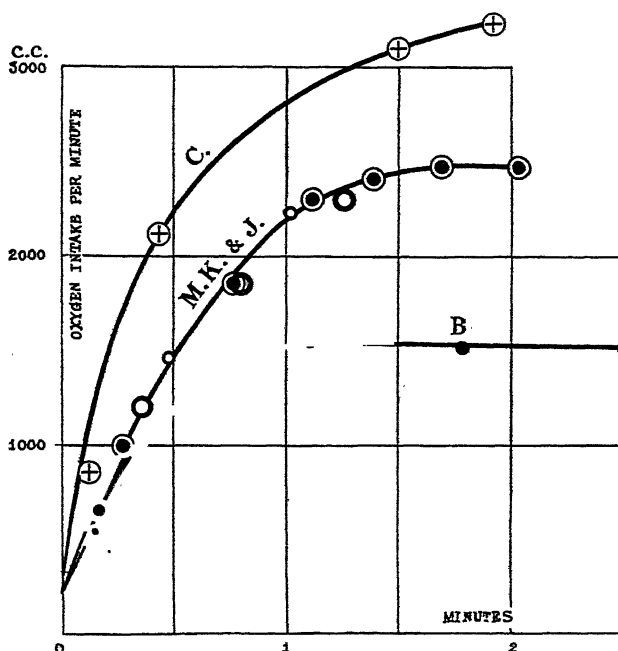


FIG. 5.—Rate of oxygen intake from the beginning of exercise: B, 140 steps per minute; M, K, and J, 180 steps per minute; C, 270 steps per minute.

two minutes. These curves are exactly similar in character to those given by Hill and Lupton.

4. *The whole period of exercise.*—Fig. 6 shows one set of results obtained on a

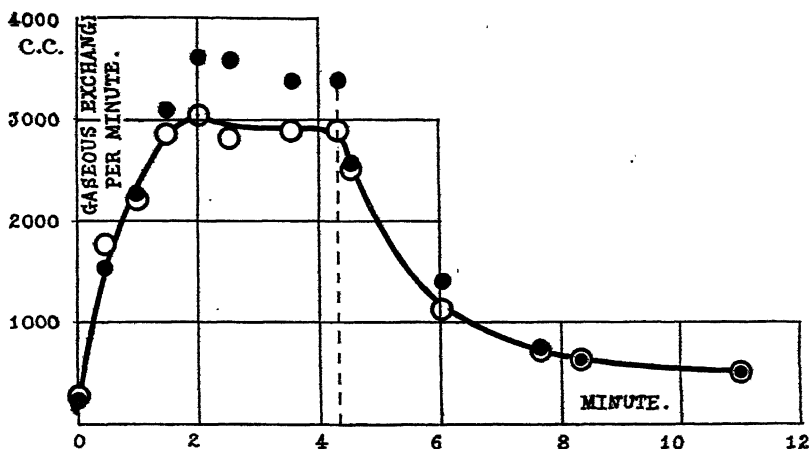


FIG. 6.—Rate of oxygen intake (hollow circles) and of carbon dioxide output (black circles) during exercise and recovery; vertical broken line represents end of exercise.

good quarter-mile runner, performing "standing-running" and followed throughout the whole period of exercise. The expired air was switched into the spirometer at the moment when exercise began. Samples were taken continually throughout exercise and recovery, and the expired gases were switched off again only when recovery was nearly complete. The blank circles denote the oxygen intake per minute, and the black dots the carbon dioxide expired per minute. Thus, it is possible to follow the rapidly altering gaseous exchanges of exercise in a single experiment, from its beginning to the end of recovery, provided only that the exercise is not of such severity that the spirometer is full before the recovery is complete.

Summary.

A new method is described of studying continuously the rapidly altering gaseous metabolism of man during and after muscular exercise. A spirometer of large capacity is used, the gases inside it being kept continuously stirred, the total ventilation being measured every 10 litres, and samples being abstracted at intervals for analysis. Various examples are given. Results are obtained similar to those by other methods, but involving considerably less labour.

It is my sincere desire to acknowledge my indebtedness to Prof. A. V. Hill, with whose kind advice and encouragement this method has been developed.

The apparatus employed in this research has been purchased by Prof. A. V. Hill by means of a grant from the Medical Research Council.

REFERENCES.

- (1) Campbell, Douglas and Hobson : 'Phil. Trans.,' B, vol. 210, p. 1 (1920).
 - (2) Hill, Long and Lupton : 'Roy. Soc. Proc.,' B, vol. 97, p. 87 (1924).
 - (3) Hill and Lupton : 'Quart. Journ. Med.,' vol. 16, p. 145 (1923).
-

Muscular Exercise, Lactic Acid, and the Supply and Utilisation of Oxygen.—Part XIII. The Gaseous Exchanges of Restricted Muscular Exercise in Man.

By K. FURUSAWA.

(Communicated by Prof. A. V. Hill, F.R.S.—Received September 21, 1925.)

(From the Department of Physiology, University College, London.)

The type of exercise studied in former papers of this series involves the activity of the body as a whole. The characteristic of all such forms of exercise is the free and vigorous movement of nearly all the muscles in the body. Since nearly all the muscles were in activity and behaving in a similar way it was more easy to compare their behaviour with that of an isolated muscle, and in previous papers it has been shown how closely the phenomena of muscular exercise in the body as a whole resemble those accompanying severe exercise in the isolated muscle.

As far as concerns the observations described in the succeeding pages, the most pertinent conclusions of the former papers are as follows: (1) Provided that the exercise was not too severe, there occurred what has been called a steady state, in which recovery balanced breakdown in a manner analogous to that shown by Fletcher to occur in isolated frog's muscle; (2) in severe exercise a considerable proportion of the energy employed is derived, not from contemporary oxidation, but by lactic acid formation on what may be called a "credit" of oxygen secured on the oxidation occurring in the recovery process later; (3) the most severe exercise can be maintained only for about 30 seconds, which corresponds to the time when the lactic acid concentration in the active muscle, as measured by the magnitude of the oxygen debt, may reach a value of about 0.3 per cent., the maximum value found to occur in the isolated frog's muscle.

In the present paper a form of restricted exercise has been studied, to compare its effects with those of severe general exercise of the type of "standing running." The mass of the muscles used in this type of exercise is small, so that we may find the influence of the resting muscles on the active ones. The speed can be changed from very slow to the highest possible, as in "standing running." In order to fulfil these conditions a simple ergometer was devised, which involves the muscles only of the arms and shoulders. The expired gases were usually treated in a manner similar to that described in

previous sections of this series. In some of the later experiments, however, the spirometer described in the preceding paper was used.

Ergometer.

Use was made of a simple spring ergometer, having a pair of wooden levers which are moved backwards and forwards by the hands, and a pair of powerful steel springs attached to the levers. Fig. 1 represents a side elevation of this machine. The weight of the subject, together with that of the working parts

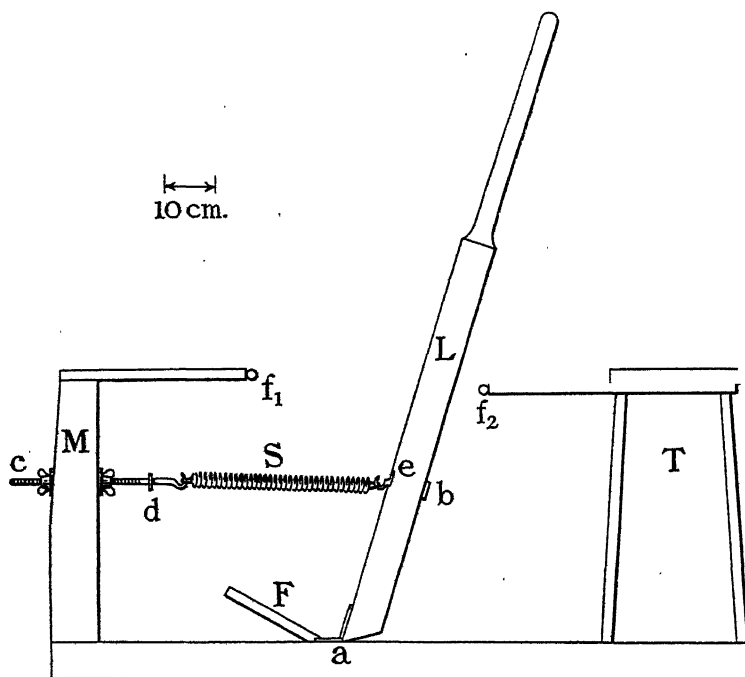


FIG. 1.—Spring ergometer. L, levers (two, one only shown); T, stool; S, steel springs; f_1 , f_2 , buffers faced with rubber tube; F, foot rest.

of the apparatus, is taken by a massive wooden frame, 140 cms. in length and 44.5 cms. in breadth. The lever L is 130.5 cms. long, and is fixed by a hinge to the wooden frame at a point a 81 cms. from the rear end—that is, the right in the figure. The lower part (84 cms.) of this lever consists of a block of wood 7 cms. square, while the upper part is cylindrical and 3.7 cms. in diameter. The spring S has a coil 3.1 cms. in diameter and 13.5 cms. in length (unstretched). It is made of steel wire 3.0 mm. thick, and finishes in a loop at each end. One of the loops is connected to a hook fixed on the lever

at b 33 cms. from a , while the other end is attached to another hook at the end of an iron bar c , passing through a hole bored in a massive wooden block M fixed on the front of the frame. The tension of the spring is adjusted by a wing-nut on the iron bar c . The range of movement of the two levers L is determined by two pairs of wooden arms attached to the top of M and to the stool T . Each arm has a buffer f of stout rubber tube, to mitigate the impact when the lever strikes the head of the arm. T is a simple laboratory stool 56 cms. high, screwed to the wooden frame. F is used as a foot-rest for the subject.

The experiment is carried out as follows: The subject rests on the chair for 10 to 20 minutes, a basal value being then taken if required. The exercise consists of pulling the levers backwards and forwards alternately, the right arm pulling the lever forward, while the left arm allows it to return, and *vice versa*. By this means, apart from a small torque on the trunk and a certain rigidity of the abdominal and back muscles, activity is limited to the arms and shoulders, and fulfils the conditions of restricted exercise discussed above.

Work Done.

In order to calculate the amount of work done by the arms it is necessary to know the tension developed when the spring is stretched. Two points d and e are fixed on the lever and on the iron bar c respectively. Fig. 2 shows

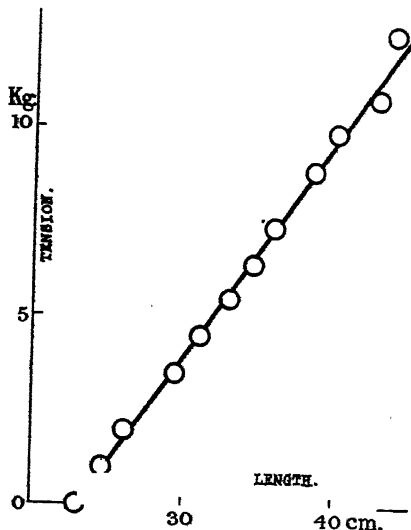


FIG. 2.—Tension in spring (vertically) as a function of its length (horizontally).

the tension t developed in the spring as a function of the distance between d and e , which is denoted by l . The force is measured at a fixed point on the lever, which is the centre of the force applied by the hand of the subject. From fig. 2 it is obvious that Hooke's law can be applied to the calculation. The work may be calculated from the formula:

$$W = \int_{l_1}^{l_2} t \, dl,$$

where l_1 and l_2 denote the distances between d and e when the position of the lever is at f_1 and f_2 respectively. The relation between t and l is given empirically by the formula:

$$l = 1.72t + 23 \text{ (kg. cm. units).}$$

From this we find the equation:

$$W = \frac{1}{2} \times 1.72 [t_2^2 - t_1^2].$$

Oxygen Requirement.

In preceding papers of this series the oxygen requirement of "standing running" and flat running have been studied as a function of speed (1). The oxygen requirement follows a simple curve concave upwards, approaching a certain limiting speed almost asymptotically. These curves gave the oxygen requirement per minute for the exercise considered. The oxygen requirement for 100 steps is not constant therefore, but increases with the speed, since a constant oxygen requirement per 100 steps would imply an oxygen requirement per minute rising uniformly with the speed and not on a curve concave upwards. More and more energy therefore is used in such kinds of exercise as the speed increases, and there is no optimal speed. In running on the flat and in "standing running" we are dealing with a type of exercise in which there is no external load, the whole effort is used in accelerating the parts of the body itself. In the present case, however, the conditions are different: there is an external load independent of the speed, and an optimal speed exists.

The study of the oxygen requirement has been made in two separate cases: one involves constant tension at varying speed, the other constant speed at varying tension. Table I shows the results obtained in the former case. The work done in each stroke is 2.41 kg.-metres.

The first two columns show the average values of (i) the basal oxygen intake and (ii) the basal CO_2 output, measured both before exercise and after recovery; the third column gives the speed, varying over a very wide range, from 12 strokes per minute (when one complete movement of each arm occupies 10

Table I.—Constant Tension and Varying Speed.

Basal value : Cubic centimetres per minute.		Speed : Number of strokes per minute.	Duration of exercise : Minutes.	Time of collection : Minutes.	Excess oxygen used per 100 strokes : Cubic centimetres.
O ₂ .	CO ₂ .				
222	181	12	4.59	21	2,290
245	215	81	1.23	18	1,421
227	171	100	1.08	15	1,297
227	193	135	1.11	20	1,076
227	196	160	0.63	15	1,023
258	208	200	0.50	15	1,018
215	173	232	0.60	20	1,059
230	182	268	0.52	23	1,380
211	175	290	0.34	20	1,878
234	190	290	0.38	20	2,165
271	205	12	5.00	16	3,666
289	232	140	0.85	22	1,318
296	248	290	0.41	33.5	2,644

seconds) to 290 strokes per minute (when 0.42 second only is used in completing the double stroke of each arm). The fourth column gives the duration of the exercise, which at the highest speed is necessarily very short since the effort involved is extremely exhausting. Column 5 shows the time of collection of the expired gases, covering both exercise and recovery. Column 6 gives the amount of oxygen required, in excess of basal, to carry out 100 complete strokes, that is 50 with each arm. The figures in this column are shown graphically in figs. 3 and 4. Fig. 3 gives the oxygen requirement for 100 strokes

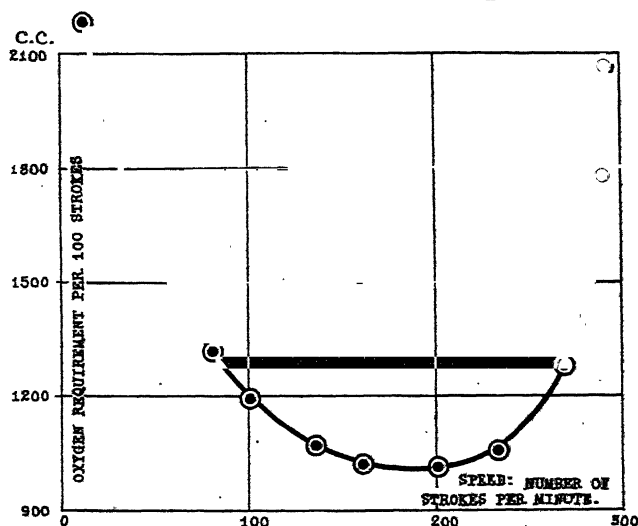


FIG. 3.—Oxygen required per 100 strokes as a function of speed; load constant.

as a function of the speed, that is of the number of strokes per minute. Fig. 4 gives the oxygen requirement per minute as a function of the speed, and also the oxygen intake. We shall refer to the latter later.

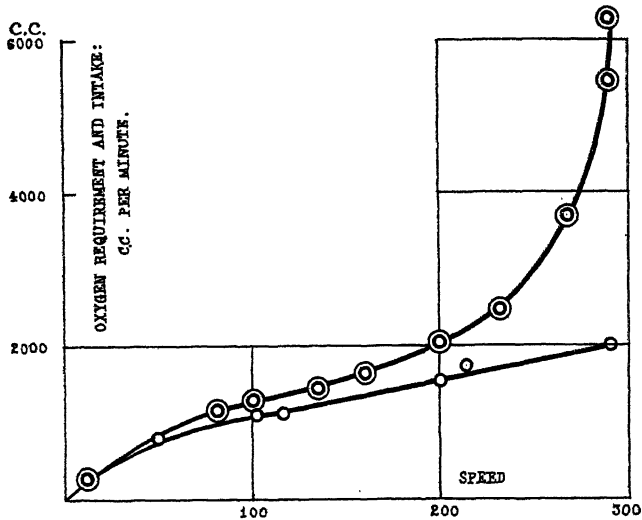


FIG. 4.—Upper curve, oxygen required per minute; lower curve, oxygen intake per minute after a suitable fore-period of exercise (see Table III); load constant.

Fig. 3 shows very clearly the extremely uneconomical nature of a movement which is, either very slow or very rapid. The oxygen requirement per 100 strokes decreases at first as the speed increases, and reaches a certain minimum value, at which about half as much oxygen is used as at 12 strokes per minute. The oxygen requirement then increases gradually as the speed further increases, and later more rapidly, attaining a very high value at the maximum speed of 290 strokes per minute. In Table I the first ten results refer to the subject K.F., the last three to the subject R.A., who is a practised sculler. The latter subject shows the phenomenon even more strikingly than the former.

It is interesting to compare fig. 4 with the curves given in a previous paper (1) for the cases of running and "standing running." There the curves were concave upwards throughout their course; here it is initially convex and finally concave, the point of inflexion of the curve corresponding to the optimal speed. The S-shape of the oxygen requirement curve of fig. 4 is a sign of the existence of an optimal speed. This does not occur in exercise of the type of running or "standing running," where there is no external load independent of the speed of movement.

If we assume that the relative efficiency of the muscular exercise can be expressed as the reciprocal of the oxygen used to perform a given amount of

work, then we may calculate a curve of efficiency in this particular exercise which is similar to that obtained by Lupton (2) for the case of stair-climbing. Lupton found the optimum to occur when a single contraction occupied about 1.3 second. In the case investigated here the optimum speed is considerably higher, namely, that at which each arm completes 100 movements every minute. The cause of this difference is not known; possibly it is due to the fact that in the type of exercise investigated here lengthening of the muscle as well as shortening takes place under a load.

The case of variable load at constant speed also has been investigated. The constant speed employed was 142 strokes per minute, not very far from the optimum, and the load was varied by changing the tension in the spring. The results are given in Table II.

Table II.—Constant Speed and Varying Tension.

Basal value: Cubic centimetres per minute.		Duration of exercise: Seconds.	Time of collection: Minutes.	Oxygen re- quirement per 100 strokes: Cubic centimetres.	Work done in each stroke: Kg. cm.
O ₂ .	CO ₂ .				
210	165	84	15	199	0.0
247	201	42	10	232	0.0
221	191	84	20	616	130
198	161	42	15	690	147
220	—	42	15	1,005	156
206	167	42	17	850	165
221	189	84	30	1,015	170
239	197	42	15	797	170
240	207	42	18	662	192
241	194	42	16	1,064	224
229	183	42	15	835	224
234	192	42	15	1,190	241

The first four columns explain themselves; the fifth column is the oxygen requirement per 100 strokes, and the last column shows the work done by the arms in each stroke. Fig. 5 gives the oxygen requirement per 100 strokes as

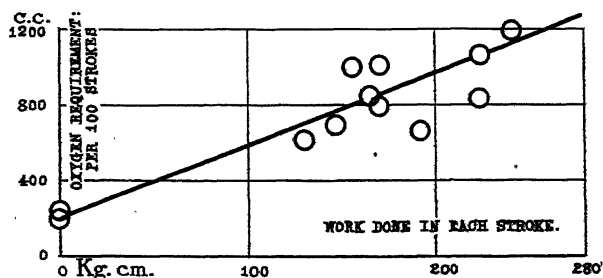


FIG. 5.—Oxygen required per 100 strokes, at constant speed but variable load.

a function of the work done. If we subtract the oxygen requirement of moving the arms at the given speed without load, we may conclude from fig. 5 that the efficiency of muscular movement in this particular type of exercise is constant at the value of 14 per cent. approximately, and independent of the amount of work done. Thus, although there is an optimal speed, there is no optimal load, the efficiency for a given speed being independent of the load.

The absolute value of the efficiency, namely, 14 per cent., is low compared with the results obtained by other investigators on other types of exercise, in spite of the fact that the subject was working at a speed not far from the optimal. This fact may, no doubt, be attributed to the nature of the ergometer employed, in which the effort of the muscle in absorbing work during the return stroke is entirely wasted so far as a determination of the efficiency goes.

Oxygen Intake and Oxygen Debt.

The oxygen intake curve of exercise involving most of the muscles of the body, such as running or "standing running," rises at once when the exercise is started, and reaches a steady state usually in about two minutes or less. The oxygen intake, once the steady state is attained, corresponds closely to the oxygen requirement provided that the exercise is well within the capacity of the individual concerned.

Table III.—Oxygen Intake and Speed.

Speed: Number of strokes per Minute.	Foreperiod of exercise: Minutes.	Time of col- lection: Minutes.	Ventilation: Litres per minute.	Oxygen intake: Cubic centimetres per minute.
50	5.0	1.0	21.7	811
102	2.5	1.0	28.5	1,105
116	2.5	1.0	34.0	1,220
200	3.0	2.0	46.7	1,531
214	2.0	1.0	57.8	1,734
290	2.0	1.0	66.0	2,013

In Table III. the values of the oxygen intake are given for the ergometer exercise, usually after two to three minutes of previous exertion. These values are those plotted in fig. 4, and referred to above. It is here that there is a divergence between the oxygen requirement, and the oxygen intake, even for quite moderate exercise. It would seem, therefore, that the foreperiod of exercise allowed, 2 to 3 minutes, is not adequate to allow the true oxygen intake, corresponding to the exercise, to be attained, so that in this respect the

exercise differs from that of a general nature such as running. This possibility was tested directly, as shown in fig. 6, and found to be the case.

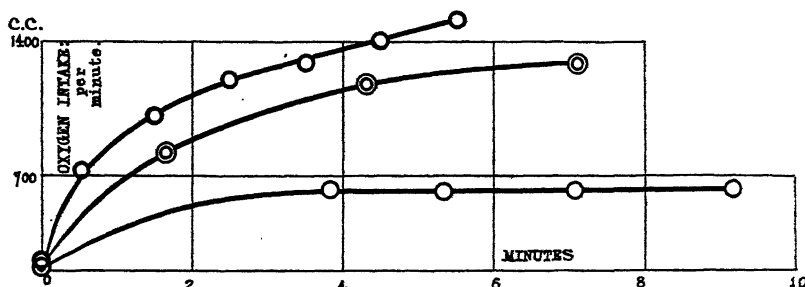


FIG. 6.—Rate of oxygen intake starting from the beginning of work; lowest curve very moderate exercise; upper curves more severe exercise. (For time relations compare fig. 5 of preceding paper.)

In very moderate exercise, represented by the lowest curve, the oxygen intake attained a steady state in about 3 minutes. In the upper curves, however, the state of affairs is different; they continue to rise even after 6 minutes. This characteristic of the oxygen intake suggested that this type of exercise might cause a large oxygen debt, if continued long enough, which might be comparable to that resulting from exercise of the body as a whole. Table IV. shows some observations made at a speed of 180 strokes per minute, the work done in each stroke being 2.24 kgm. metres.

Table IV.—Oxygen Debt and Duration of Exercise.

Basal value : Cubic centimetres per minute.		Duration of exercise : Minutes.	Time of collection : Minutes.	Oxygen debt : Cubic centimetres.
O ₂	CO ₂			
253	208	1.5	29.5	1,335
233	206	3.0	45.0	3,835
251	221	3.0	40.0	4,686
233	200	4.0	60.0	5,892
266	225	5.0	60.0	8,431

From the last column of this table it is clear that the oxygen debt increases as the duration of the exercise increases, attaining a value of 8.5 litres after 5 minutes of exercise.

These apparently anomalous facts, the slow rise of the oxygen intake and the large oxygen debt corresponding to exercise of only a few muscles, has probably quite a simple explanation, one, however, which is of the greatest importance in the general physiology of muscular exercise. If we may suppose that when only a few muscles of the body are in extremely active movement,

a part of the lactic acid formed in them is carried by the blood stream away to other parts of the body, *e.g.*, to the resting muscles and the liver, we have a complete explanation of the phenomena discussed above. In the resting muscles and the liver the lactic acid formed in the active muscles may presumably be eliminated by a process similar to that occurring in an active muscle. The interpretation of the results given above is strongly supported by the observations of Meyerhof (3), who has found that the resting isolated muscle has a capacity to transform lactates in the Ringer's solution around it to glycogen inside it. It is also confirmed by the observations of Barr, Himwich and Green (4), who found that in the strenuous exercise of one localised group of muscles the venous blood going to resting muscles might contain more lactic acid than the arterial blood.

In the case of the restricted exercise studied here the oxygen intake cannot rise very far because the oxygen supply to the active muscles is necessarily limited by the amount of blood which can be brought to them through the circulation. This oxygen, when the exercise is very severe, is in no way adequate to the needs of the muscles; they liberate lactic acid far in excess of any power of restoring it to glycogen which they themselves possess. The lactic acid, therefore, begins to accumulate within them, but under its concentration gradient it rapidly passes into the blood stream and is carried round the body. From the blood it passes into other organs, which then commence the oxidative restoration of the lactic acid to glycogen, so that the oxygen intake gradually rises, as the lactic acid formed in the active muscles is transferred for removal to different parts of the body. The process presumably can go on until the whole of the lactic acid precursor in the active muscles has been used up and transformed, partly into CO_2 and water, but mainly into glycogen in other tissues of the body. It would be very interesting to study this type of muscular exhaustion. It is quite different from any of which we are aware in the body as a whole.

A brief calculation reveals an interesting confirmation of this point of view. The following table shows the results obtained on two subjects carrying out

Subject :	Speed : Number of strokes per minute.	Body weight : Kg.	Maximal duration of exercise : Seconds.	Weight of muscle used : Kg.	Oxygen requirement per 100 strokes : Cubic centimetres.
R.A.	290	65	24	7.67	2,644
K.F.	290	47	23	5.6	2,165

the same number of strokes of very severe exercise at the same speed. In the last column is given the extra oxygen used to carry out the exercise, which was so severe that neither subject could continue it any longer than the 23 or 24 seconds shown in column 4. Presumably the exercise had to terminate because the lactic acid had accumulated to its fatigue maximum in the active muscle. If we assume that 1 litre of oxygen debt corresponds to 7 gms. of lactic present in the muscle at the end of exercise, and that the fatigue maximum is 0.3 per cent., then the weight of the muscles used may be calculated as shown in column 5. The ratio of the body-weight of the two subjects is 1.38, and that of the active muscles, as calculated in column 5, is 1.37. The agreement suggests that we are approximately correct in our calculation of the mass of the active muscles of K.F., for this type of exercise, as being 5.6 kg. An oxygen debt of 8.4 litres, however, requires about 19.5 kg. of muscle to accommodate lactic acid in 0.3 per cent. solution. This means that exercise at 180 strokes per minute prolonged for 5 minutes involves the saturation to the fatigue maximum of $3\frac{1}{2}$ times as much muscle as is used during the exercise. Actually, of course, the lactic acid corresponding to the oxygen debt is probably distributed over all the tissues of the body, and the fatigue maximum is not attained in any of them except those which are actually working. It is striking, however, to find that quite severe general fatigue might be produced in the human body by violent exercise involving only such a small fraction of its total musculature.

Summary.

1. The gaseous metabolism has been studied for a type of exercise involving only a limited amount of the musculature of the human body.
2. For this purpose a simple ergometer, capable of being worked at any speed by the arms, has been constructed.
3. The oxygen required to perform a given amount of work varies with the speed at which the work is done. There is a marked optimal speed.
4. On the other hand, with constant speed and varying load, the oxygen requirement rises as a linear function of the work done, and there is no optimal load.
5. The oxygen intake rises much more slowly to its maximum value than it does in the case of exercise involving most of the muscles of the body, *e.g.*, running or "standing running." The oxygen intake may be still rising rapidly after 6 minutes of exercise.

6. Corresponding to this the oxygen requirement curve diverges at quite low speeds from the oxygen intake curve, while a large oxygen debt may be set up by a few minutes' exercise involving only a small fraction of the total musculature of the body.

7. These facts indicate that the lactic acid produced in excess by violent activity of a localised group of muscles may diffuse from them into the blood, and thence to other parts of the body, particularly the resting muscles and the liver, and there be removed or restored to glycogen, under the influence of oxidation occurring in those other tissues.

8. There would appear, therefore, to be the possibility of a type of exhaustion due to complete using up of the lactic acid precursor, when the exercise involved is localised in a small group of muscles. The function of the circulation in distributing the fatigue product, lactic acid, to other organs of the body which are themselves at rest would seem to be an extremely important one from the point of view of the vigorous activity of local groups of muscle.

In conclusion it is a great pleasure to express my heartiest thanks to Prof. A. V. Hill for his suggestion of these experiments, and for encouragement given to me during their conduct.

The expenses of this research have been borne in large part by a grant, placed at my disposal through Prof. A. V. Hill, by the Medical Research Council.

REFERENCES.

- (1) Furusawa, A. V. Hill, Long and Lupton, 'Roy. Soc. Proc.,' B, vol. 97, p. 155 (1924).
 - (2) Lupton, 'Journ. Physiol.,' vol. 57, p. 337 (1923).
 - (3) Meyerhof, Lohmann and Meier, 'Biochemische Zeitschr.,' vol. 157, p. 459 (1925).
 - (4) Barr, Himwich and Green, 'Journ. Biol. Chem.,' vol. 55, p. 525 (1923).
-

Muscular Exercise, Lactic Acid, and the Supply and Utilisation of Oxygen.—Part XIV. The Relation in Man between the Oxygen Intake during Exercise and the Lactic Acid Content of the Muscles.

By C. N. H. LONG.*

(Communicated by Prof. A. V. Hill, F.R.S.—Received October 8, 1925.)

(From the Department of Physiology, University College, London.)

Introduction.

Hartree and A. V. Hill (1) have shown in the isolated frog's muscle that the maximum rate of the recovery heat-production is proportional to the square of the total initial heat liberated by the stimulus. This relation has been amply confirmed by Hartree, in experiments hitherto unpublished on the isolated muscle of the tortoise. Hartree and Hill concluded that the speed of the recovery oxidation is at any moment proportional to the square of the concentration of the reacting bodies present at that moment, and suggested that a bimolecular reaction of some kind, possibly involving two lactic acid molecules, might be the determining agent in the velocity of the recovery oxidation. Up to the present it has not been shown that any substances other than lactic acid and oxygen are directly concerned in the recovery process in muscle, so the above relationship can be put in terms of these substances. Expressed in a formula,

$$(\text{rate of oxidation}) \propto (\text{rate of lactic acid removal}) = k (\text{concentration of lactic acid})^2,$$

where k is a velocity constant.

In a recent paper Meyerhof, Lohmann and Meier (2) have shown that isolated frog's muscle perfused by, or suspended in, an oxygenated Ringer's solution containing sodium lactate will cause a diminution in the amount of lactate in the perfusing fluid, with an uptake of oxygen and a synthesis of glycogen. In these experiments it was obvious that the lactate ion was the determining factor in exciting the oxidation by which the energy was supplied to effect the synthesis of the glycogen. Moreover, as Hartree and Hill have shown (3), a rise of hydrogen ion concentration alone does not increase, but rather

* Working on behalf of the Industrial Fatigue Board, Medical Research Council, to whom also I am indebted for a grant for apparatus.

decreases, the speed of the recovery process in muscle. It seemed obvious, therefore, that the lactate ion itself was to be regarded as the governing factor in the speed of oxidation.

In the experiments to be described I have attempted to show that the same relation holds good in man. The oxygen intake during a steady state of exercise has been taken as a measure of the rate of lactic acid removal, and the lactic acid content of the blood plasma as being equal to the lactic acid concentration in the fibres. For the latter assumption to be justifiable it is necessary that the body should be in a steady state of exercise, the effort involved having been continued a sufficiently long time for that state to have been reached. The absolute size of the oxygen intake will vary with the degree of effort involved, and for each different value of the rate of oxygen intake there should be a definite value of the lactic acid concentration in the muscle, the relation between the two being such that an increase in the oxygen intake is accompanied by an increase in the lactic acid concentration, not directly proportional to the oxygen intake, however, but only to its square root.

Methods.

All these experiments were performed on healthy young men. The exercise has been either walking or standing running at different speeds, except in one experiment, where the subject (a Marathon runner) was engaged in a training run of 15 miles. In every case the subject was in a steady state—that is, lactic acid formation was balanced by lactic acid removal, so that the lactic acid in the blood had attained a constant concentration. It is essential that this should be so, for otherwise, if the oxygen supply were inadequate, the lactic acid would accumulate in the muscle and appear in excess in the blood. Values up to 0.2 per cent. have been recorded (4) in the blood of subjects after severe exercise, whereas, if the subjects are in a steady state of exercise, the lactic acid concentration of the blood should never exceed about 0.06 per cent.

To ensure that the subjects were in a steady state, and that the lactic acid concentration of their blood plasma approximated more or less to that in the muscle, the exercise in all experiments was continued for some 20 to 30 minutes. Before commencing, the subject's resting respiratory exchange was measured by the Douglas bag method, and a sample of blood withdrawn from a vein in his arm. He then commenced the exercise and continued it for 20 minutes or more. His respiratory exchange during exercise was then determined by collecting his expired gases in a small Douglas bag which he had previously carried in his

hand. Immediately after this the second blood sample was taken. The lactic acid was estimated by Clausen's method (6).

Results.

It is necessary to apply certain corrections to the lactic acid concentrations observed. Firstly, the lactic acid content of the whole blood is not the same as that of blood plasma. It has been shown (4) that the ratio of the content in the plasma to that in the whole blood is 1.28. Furthermore, I have found (5) that only two-thirds of the lactic acid content of the resting blood as measured is actually lactic acid itself, the rest being other substances, which yield bisulphite binding compounds on oxidation. To determine, therefore, the true lactic acid concentration of the blood at rest one-third of the value given by Clausen's method must be subtracted, and to convert the remainder into the true value for the blood plasma this corrected value has then been multiplied by 1.28. In the case of the lactic acid concentration of the blood during exercise we have to assume that the quantity of substances, other than lactic acid, which are estimated as lactic acid, is unchanged as the result of exercise. The substances responsible for the error are probably chiefly amino acids and phenols, and it seems reasonable to assume that large variations in the quantities of these bodies will not occur as a consequence of what, in the majority of experiments, is only mild exercise. The exercise lactic acid has been corrected therefore by the same absolute amount as the resting lactic acid, and the remainder has been multiplied as before by 1.28 to obtain the lactic acid content of the plasma. While we know that the blood plasma itself is not in actual contact with the muscle fibres, yet after a long period of steady exercise its lactic acid content must approach that of the lymph which is.

Thirteen experiments have been performed on eight different subjects: the results are given in Table I below. In all the experiments, except No. 5, the agreement between the measured relative increase of the lactic acid content of the blood plasma, and the relative increase as calculated by the square root from the relative increase in oxygen intake, is fairly good. In Experiment 5 the lactic acid content of the plasma is far too great for the degree of exercise which was being performed. It would seem that some error of unknown origin crept into this estimation.

The results are best expressed graphically. In the diagram (p. 171) the relative increase in the lactic acid content of the plasma has been plotted against the relative increase in the oxygen intake. Along with the observed points two theoretical curves have been drawn: the broken line expresses the relation

Table I.

Subject and exercise performed.	Resting oxygen intake c.c./min.	Oxygen intake during exercise, c.c./min.	Relative increase in oxygen intake.	Corrected lactic acid in blood plasma (mgms./100 c.c.).		Relative increase in lactic acid.	
				Rest.	During exercise.	Measured.	Calculated.
(1) C.N.H.L. Standing running at 156 s.p.m. for 45 min.	287	2038	7.10	17.0	33.6	1.98	2.66
(2) C.N.H.L. Walking at 4.1 m.p.h. for 30 min.	294	2340	7.96	12.9	40.0	3.11	2.82
(3) C.N.H.L. Walking at 3.5 m.p.h. for 30 min.	269	1660	6.17	15.6	36.0	2.30	2.49
(4) C.N.H.L. Walking at 3.5 m.p.h. for 30 min.	249	1155	4.64	17.9	37.9	2.12	2.16
(5) C.N.H.L. Walking at 4.1 m.p.h. for 30 min.	282	1241	4.40	18.3	66.5(?)	3.64	2.10
(6) J.C.H. Walking at 3.3 m.p.h. for 20 min.	285	906	3.18	27.5	38.5	1.40	1.78
(7) J.C.H. Walking at 3.3 m.p.h. for 30 min.	244	1595	6.55	17.4	40.0	2.30	2.56
(8) A.C.K. Standing running at 180 s.p.m. for 24 min.	324	1670	5.15	14.0	28.3	2.02	2.27
(9) D. Standing running at 185 s.p.m. for 31 min.	352	1352	3.85	19.7	36.7	1.86	1.96
(10) K.F. Walking at 3.7 m.p.h. for 32 min.	263	1170	4.45	14.9	36.2	2.43	2.11
(11) S.S. Walking at 3.5 m.p.h. for 30 min.	312	1250	4.00	20.1	43.0	2.14	2.00
(12) P.E. Walking at 4.5 m.p.h. for 25 min.	253	2060	8.15	15.6	38.5	2.47	2.86
(13) M.R.D. Running at 8½ m.p.h. for 100 min.	321	3165	9.85	17.9	70.0	3.81	3.14

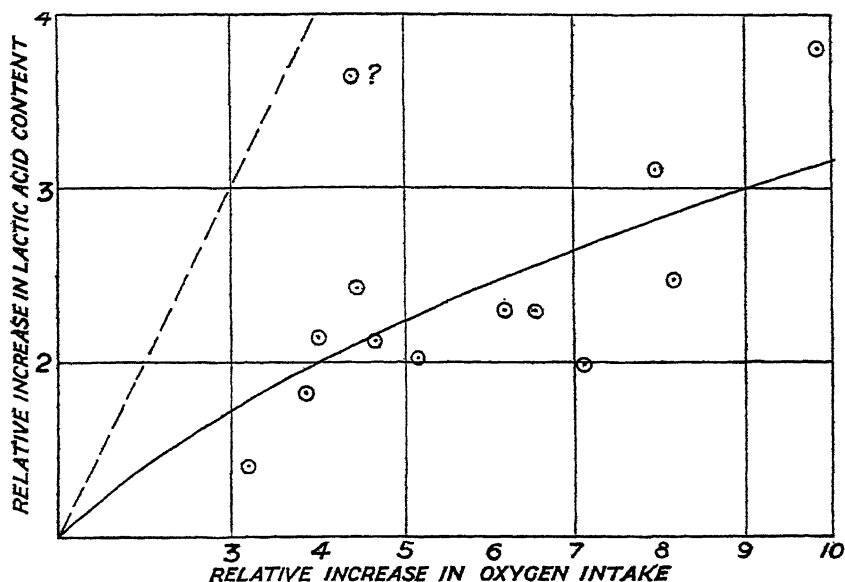
Note.—s.p.m. = steps per minute; m.p.h. = miles per hour.

which would exist between these two quantities if one were proportional to the other—that is, if the recovery process were governed by a monomolecular reaction, with the formula

$$(\text{oxygen intake}) \propto (\text{rate of lactic acid removal}) = k (\text{concentration of lactic acid}).$$

The full curve shows the relation which would be found if the oxygen intake varied as the square of the lactic acid concentration, according to the formula on p. 167 above. It is clear that the relation established experimentally between the oxygen intake and the lactic acid concentration is of the second type. The observed points fit the full curve in the figure quite reasonably well, when due account is taken of the relatively large errors which must

necessarily affect such experiments as these. This result is in complete agreement with that found by Hartree and Hill for the case of isolated muscle recovering from a single stimulus.



Experimental observations shown by circles. Broken curve is the theoretical relation for a monomolecular reaction. Full curve is that for a bimolecular reaction.

Discussion.

If this relation be a true one, then it follows that in the recovery process in muscle lactic acid plays the important part of a "governor of oxidation." This term is employed from the analogy of a steam engine, where a centrifugal governor, in which the centrifugal force varies as the square of the velocity, regulates the rate at which energy is allowed to be liberated. Up to a certain limit the greater the rate of formation of lactic acid the faster will be the rate at which it is removed, the upper limit of the rate of removal being set by the maximum oxygen intake of the subject. In the absence of an adequate supply of oxygen, lactic acid will accumulate in amounts far in excess of that required for the maximum oxygen utilization of the muscle. In normal men the oxygen intake may increase—at any rate, in athletic subjects—about 16 times, *e.g.*, from 250 c.c. to 4000 c.c. per minute. Corresponding to this there will have to be a fourfold increase in the lactic acid content of the muscles or blood plasma, *e.g.*, from 0.02 to 0.08 gms. per 100 c.c. The maximum concentration of lactic acid that can be attained in human muscles as the result of violent

exercise in the absence of a sufficient supply of oxygen—that is, when the effort exceeds that corresponding to a steady state—is far higher than this, being probably about 0.3 to 0.4 per cent. (4). This means that the muscle can go on accumulating lactic acid till its content is four to five times that required to ensure the maximum rate of oxygen usage.

As to the precise nature of the chemical reactions which constitute the recovery process, these experiments on man cannot throw very much light. Further knowledge of the intimate nature of the process will probably come from a renewed study of the isolated muscle. The fact that the relation found is that corresponding to a bimolecular reaction means only that of all those reactions, oxidative and non-oxidative, that occur in the re-synthesis of carbohydrate from lactic acid, some one, the slowest of the series, is of a bimolecular nature, and it will be this particular change which imposes its own order of reaction on the whole series.

Summary.

(1) Experiments have been performed on men to test the validity of the relation found to exist, by Hartree and Hill, in the case of the isolated muscle, between the speed of the oxidative removal of lactic acid in recovery and its concentration in the muscle at the same time.

(2) In men, as well as in the isolated muscle, it has been found that the rate of removal of lactic acid, as measured by the oxygen intake, is proportional to the square of the lactic acid concentration in the fluids which are in contact with the muscle fibres.

(3) Lactic acid apparently acts as a “governor of oxidation” in the recovery process of muscle.

My best thanks are due to Prof. A. V. Hill for suggesting this problem and for much help and advice during its investigation.

REFERENCES.

- (1) Hartree and A. V. Hill, ‘Journ. Physiol.’ vol 56, p. 367 (1922).
- (2) Meyerhof, Lohmann and Meier, ‘Biochem. Zeitsch.’ vol. 157, p. 459 (1925).
- (3) Hartree and A. V. Hill, ‘Journ. Physiol.’ vol. 58, p. 470 (1924).
- (4) Hill, Long and Lupton, ‘Roy. Soc. Proc.’ B, vol. 96, p. 438 (1924).
- (5) Long, ‘Journ. Physiol.’ vol. 58, p. 455 (1924).
- (6) Clausen, ‘Journ. Biol. Chem.’ vol. 52, p. 263 (1922).

The Hydrogen-Ion Concentration and Oxidation-Reduction Potential of the Cell-Interior before and after Fertilisation and Cleavage: A Micro-Injection Study on Marine Eggs.

By JOSEPH NEEDHAM, M.A., Ph.D., Fellow of Caius College, Cambridge, and
DOROTHY MOYLE NEEDHAM, Girton College, Cambridge, Beit Memorial
Research Fellow.

(Communicated by Sir Frederick Hopkins, F.R.S.—Received October 9, 1925.)

(From the Biochemical Laboratory, University of Cambridge, and the Marine Biological
Station of Roscoff, University of Paris.)

CONTENTS.

	PAGE
Introduction	173
General Methods	174
1. The Injection Technique	174
2. The Indicators	177
3. The Material	180
Experimental Results	180
A. Staining Experiments	180
B. Determination of the internal pH by Micro-injection	181
C. Determination of the internal rH by Micro-injection	188
General Discussion.....	195
Summary.....	198

Introduction.

In our previous communications on this subject we have described experiments dealing with the micro-injection of indicators into single cells. In the conclusion to our paper (10a) on the pH and rH of the Amoeba, we said, "It is hoped that other biological data will soon be available . . . such problems as the . . . oxidation-reduction potentials of egg-cells before and after fertilisation at once present themselves." The present paper is devoted to these problems.

Warburg (17) and Meyerhof (8), and afterwards other workers, observed an enormous increase in the oxygen-consumption of the egg to take place on fertilisation. Shearer (12) found that this occurred at the moment of contact of the spermatozoon with the egg membrane. In view of the fact that the

increase was about 2000 per cent., it was clearly a matter of great interest to determine whether the rH changed at the same time. We have attempted to follow the changes in rH by micro-injection experiments and by staining. The two methods failed to give concordant results for reasons which are discussed in the text.

GENERAL METHODS.

1. *The Injection Technique.*

The micro-manipulator apparatus and micro-injection method applicable to *Amœbæ*, were discussed in our preceding paper. All that is needed here, therefore, is an account of the modifications called for when marine eggs are used. For the preparation of the micro-pipettes, a spirit lamp, having only one strand of wick passing down through a thick glass tube, was used. With this we obtained a distinctly higher proportion of good pipettes than when the hypodermic syringe gas-burner was being used.

The actual process of injection of such an egg as that of *Paracentrotus lividus* differs materially from that used for the amœba. In the case of the latter the procedure was to bring the amœba over the micro-pipette, raise the pipette so that it entered the amœba, turn the screw of the injection syringe a very little so as to make the injection and then withdraw the pipette. The egg, on the other hand, is not nearly so large nor is it possessed of so extensible a membrane. It is very much more fragile and only a small internal pressure is required to burst it. It was centred over the pipette, and the screw of the injection syringe then turned so as to make a pressure of dye at the tip of the micro-pipette great enough to force some out into a liquid, but not sufficiently great to force it out into the air. Then the micro-pipette was raised right into the egg, the injection made automatically and the pipette rapidly lowered. It is obvious that for this method there must be only a very thin layer of water underneath the egg, between it and the micro-pipette. We found, as a matter of fact, that the eggs were kept beautifully in place by the surface tension of an exceedingly thin film of water. We shall mention again the importance of this.

It is, however, not always easy to inject unfertilised eggs. The eggs of sea-urchins and of *Ophiura* gave no special difficulty, but the eggs of *Asterias* resemble a mass of porridge enclosed in a weak rubber envelope. If one tries to inject these eggs with a blunt pipette, the pipette as it rises causes an indentation which can be carried right up to the coverslip. Then the two sides of

the egg join so as to form an object exactly like a quoit, and in the centre hole a certain quantity of dye remains when the pipette is withdrawn. To the inexperienced observer this might appear to be a vacuole. This phenomenon may also happen with sea-urchin eggs, but it occurs frequently with those of *Asterias*. The eggs of *Ascidia* are an altogether different matter, being surrounded not only with a membrane, but also with a coat of tiny cells. This can easily be pierced with a sharply-pointed micro-pipette, but it gives considerable strength to the whole affair. The eggs of *Sabellaria* are only about one-fifth the size of those of *Asterias* and they possess exceedingly firm membranes.

But once fertilisation has taken place and the fertilisation membrane has risen, there are greater difficulties: then for good injections it is absolutely essential to have a pipette which is finely pointed, tapering rapidly, and sharp at the tip. Otherwise, the membrane visibly wrinkles in and no injection is made. We found it convenient to have large numbers of cells on one coverslip, since they can be had in greater abundance than can amœbæ, and consequently, it is quite a usual thing for 100 to 150 eggs to be injected on one coverslip with one micro-pipette.

The importance of having an exceedingly thin layer of water supporting the cells on the under surface of the coverslip by its surface tension cannot be over-emphasised. It is on the one hand essential for the injection procedure described above, and also important from a colorimetric point of view. The layer of water, though probably about three-quarters the thickness of the eggs between the eggs, must be very thin indeed actually below them, and therefore any colour which escapes into it must be in so thin a layer as to be invisible. A striking demonstration of this is seen if some concentrated dye be allowed to escape from the micro-pipette into one of the spaces between the eggs; a number of colourless eggs can then be seen standing out on a deep blue background.

It is important not to let the sea-water on the coverslip containing the eggs become at all concentrated. Dyes are at once precipitated in crystals if this is allowed to occur, and although the first injection may be all right the pipette will surely be blocked at the tip with crystals before a second one can be done.

In the filling of the micro-pipette the following procedure was found useful. When it is impossible to avoid very minute particles in the solution of dye, the pipette can be brought up into the drop of dye right against the coverslip, so that it bends slightly. The microscope stage is now moved slowly in an

opposite direction, so that the pipette end describes a path exactly like that of the antenna of a tramcar on the overhead system. At the same time a gentle and continuous suction is applied to the injection syringe. Particles are drawn towards the mouth of the micro-pipette, but as it is travelling as fast as they are they do not go in. By this means it is often possible to get a clear injection-liquid under unfavourable circumstances.

There are various other points which are worth noting. Sometimes it is useful to follow the suggestion of Tschautine (14)—that is, to have the pointer in the eyepiece directed to the spot where the micro-pipette is going to appear. When a large number of eggs are to be injected at once, this saves the time taken in focussing up and down to find the pipette point below the coverslip. Sometimes, as in cases where the dye which is being used stains the cell and obscures the results of micro-injection, the pipette may be brought up beside the injected cell and any dye which has escaped into the drop may be sucked back into the pipette. Or the pipette may be used as a needle, and with it the injected cell may be pushed away from the neighbourhood of the escaped dye. Also a stream of colourless sea-water may be made to flow past the cells with the same end in view. There are occasions also in which a wide-mouthed pipette may be of service. When it is desired to observe the effects of a very slow injection, a wide-mouthed pipette with sharp edges may be chosen and the dye brought to a low pressure at the tip of it. If then it is brought up against the bottom of the cell, so as to cause a deep indentation, it is found that very tiny cuts are made in the membrane round the part where the pipette is, and through these small amounts of dye leak into the interior.

The exact degree of injury caused by the actual piercing of the egg-membrane by the needle is a matter worthy of some consideration. It is important, because the method of micro-injection gives different results from certain other methods which have been employed to measure the pH of the cell-interior, and everything depends on the amount of injury which the method causes to the cell. Here it is worth-while mentioning the results of some experiments with *Asterias* eggs which were undertaken for another purpose. A considerable number (190 unfertilised and 130 fertilised) were injected with brom-cresol purple. The pipette was a good one, but the syringe was not working at its best, with the result that many of the eggs were pricked by the pipette as many as five or six times before an actual injection was made. In spite of this fact there was never any difference in the colour which this indicator took up when it did get in. It was always purple. Now since injury and

cytolysis invariably produce a burst of acidity, this meant that the pricking had not injured the cell, and that egg-cells could therefore be compared to amoebæ, which, as we showed in our previous paper, were quite unharmed by pricking. We shall return to this question when we come to consider the effects of cytolysis on the internal pH of egg-cells, and the work of Vlès and his collaborators with the method of micro-compression (15, 16).

Explosions seemed to occur more rarely with eggs than with amoebæ, and appeared to be due to some obscure type of mechanical injury, for they took place with all dyes indiscriminately.

We used throughout a Watson holoscopic eyepiece $\times 10$, and as objective either a Leitz 6.3 mm. or a Spencer 4.0 mm. Our Leitz double-eyepiece had magnification of 0. In certain cases we found dark-ground illumination to be very helpful. This was especially the case with the eggs of *Sabellaria*, which, unlike all the others we worked with, contained a considerable amount of dark granular material obscuring the colours. Here, if out of a field of eggs some were injected with No. 6 and the light shut off, the eggs stood out like blue and white counters. This technique allowed us to see whether the dyes were reduced or not in these eggs. We checked it on more transparent eggs of known behaviour, such as those of *Asterias*, and found that we obtained the same results as with transmitted light.

In all cases eggs were injected in sea-water at pH 8.4, unless otherwise stated.

2. The Indicators.

The indicators which we used for measuring the pH of the cell-interior need little comment. Our specimens of brom-thymol blue, brom-cresol purple, methyl red, and brom-phenol blue were all manufactured by British Drug Houses, while our neutral red was a pre-war specimen of Grüber's make. The concentrations which we used were as follows: 0.8 per cent. for brom-thymol blue, 1 per cent. for brom-cresol purple, 0.2 per cent. for neutral red, and for methyl red as strong an aqueous solution as could be obtained.

The oxidation-reduction potential indicators, however, merit longer description. In our work on the amoeba, we used only sodium 1-naphthol, 2-sulphonate indophenol, a specimen of which was made for us by British Drug Houses, and it will be remembered that by good chance the pH of the amoeba fell within the range of this indicator. Since then, however, we have been supplied through the great kindness of Prof. Mansfield Clark, of Washington, with eight other indicators of the pH scale, whose names are as follows in Table I and whose positions are set out on the appended diagram.

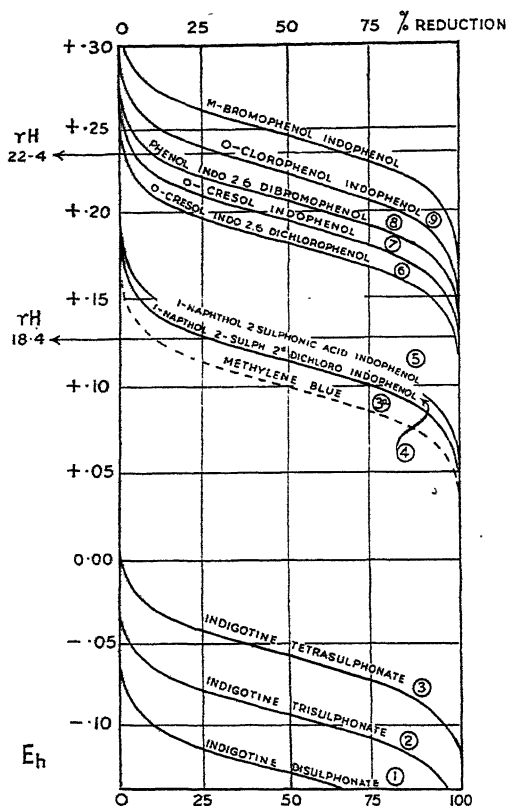


Table I.

1. Potassium indigotine disulphonate.
2. Potassium indigotine trisulphonate.
3. Potassium indigotine tetrasulphonate.
4. 1-Naphthol 2-sulphonic acid, 2, 6-dichloro-indophenol.
5. 1-Naphthol 2-sulphonic acid indophenol.
6. o-cresol, 2, 6-dichloro-indophenol.
7. o-cresol indophenol.
8. 2, 6-dibromo-phenol indophenol.
9. o-chloro-phenol indophenol.
- 3A. Methylene blue.

It will be noted that each of these indicators has been given a number ; it is by these numbers that they will hereafter be referred to. They are arranged in the figure to show their reduction curves and their positions on the electrode potential scale at pH 7.0.

We do not propose to repeat in this communication an exposition of the meaning of the term rH and the significance of oxidation-reduction potential in general; we would refer in this connection to our preceding papers (10a and b) and to those of Clark and his collaborators (1, 2, 3, 4). We need only say here that by observing which of these indicators are reduced in the cell and which are not, we are enabled to calculate its oxidation-reduction potential if we know its pH .

Needless to say, the validity of the calculation rests upon the assumption that the fundamental conception of oxidation-reduction potential can be applied to the systems of the living cell. At the present time there is no general agreement as regards the physical chemistry of the reduction electrode, but on any theory Clark's oxidation-reduction indicators must fall upon a scale. Their behaviour in the cell must therefore be a matter of permanent importance, in whatever way it may be interpreted and in whatever terminology it may be expressed. Whichever of the present theories of reduction potential, if any, ultimately turns out to be applicable to the oxidation-reduction systems in the cell, the experimental facts reported here will always require an explanation. At present they may be conveniently described in terms of rH .

The indicators for injection were used in solutions of approximately 1 per cent.

It would be legitimate criticism of our experiments to say that these concentrations and those of the pH indicators were too high to give valid results. The only answer to this objection is the simple one that if weaker indicators are used, nothing whatever can be seen under the microscope, so that the defect, if it be one, is inherent in the method. It cannot be said that dyes of such high concentration fail to show under certain conditions a pH or rH virage, because with indicator equally strong in the micro-test-tubes we could observe under the microscope a perfect gradation of colour, owing to the thinness of the layer and the strength of the light. This virage may not be identical with that seen in dilute solutions, but as the tint in the cell is compared directly with that in the micro-test-tube, this last objection has no weight.

The effect of sunlight on these rH indicators is considerable. Clark (1) warns investigators about this, and in our experience it is certainly fatal to work with solutions of the indicators which have been left standing, especially in sunlight. Our practice was to make up very small amounts of them as they were required by grinding them with water in a watch-glass, using the plunger of an old hypodermic syringe as a pestle. When this was done our results were always consistent, but we found that although normal *Paracentrotus* eggs quite failed

to reduce Nos. 2 and 3 when freshly made up, yet these indicators were reduced with ease when a solution which had been standing in the sunlight was injected.

In connection with the process of colour-comparison, it must be remembered that in practically every case the Leitz double eyepiece was employed, so that two observers noted and compared the colours. Micro-test-tubes, as described in our previous paper, were made up whenever delicate comparisons seemed necessary.

3. *The Material.*

The fertilisation of the *Paracentrotus* eggs was easily accomplished, since the spermatozoa are actively motile in the white liquid which escapes from the teased testes. This is not the case with the spermatozoa of *Asterias* however, and these have to be artificially activated by adding to a weak suspension some 0.3 N soda (2 drops to half a watch-glassful).

The eggs of *Paracentrotus* and *Echinocardium* were obtained by the method of stimulating the ripe ovaries *in situ* after opening the urchin and placing it over a beaker of sea-water. The eggs of *Asterias* and *Ophiura* were obtained by removing the ripe ovaries into a little sea-water where they spontaneously exuded. *Ascidia* eggs appear to be ripe in the oviduct only, so they were taken straight from there. *Sabellaria* eggs were easily obtained by allowing the ripe worm to lay them spontaneously in a watch-glass.

EXPERIMENTAL RESULTS.

A. *Staining Experiments.*

Before proceeding to the micro-injection of our various indicators, we thought it well to study their effect when used in the ordinary method of vital staining. *Paracentrotus* eggs were chosen as the material for this, and in the accompanying table are shown the results. The eggs were placed in sea-water at pH 8.4 containing a few drops of the indicator and left there for 30 minutes. It was found that the pH indicators did not penetrate these cells; brom-thymol blue, for example, only coloured the protoplasm green when the egg was obviously in a disintegrating condition.

Table II.

	Unfertilised eggs.	Fertilised eggs.
Neutral red	Pink.	Deep pink.
1. Potassium indigotine disulphonate	No staining.	No staining.
2. Potassium indigotine trisulphonate	No staining.	No staining.

Table II—(contd).

	Unfertilised eggs.	Fertilised eggs.
3. Potassium indigotine tetrasulphonate	No staining.	No staining.
4. 1-naphthol-2-sodium sulphonate, 2, 6-di- chloroindophenol	No staining.	No staining.
5. 1-naphthol-2-sodium sulphonate indophenol	No staining.	No staining.
6. o-cresol, 2, 6-dichloro indophenol	About 10 per cent. stained bluish-grey.	About 10 per cent. stained bluish-grey.
7. o-cresol indophenol	All stained.	All stained.
8. 2, 6-dibromo-phenol indophenol	All stained.	All stained.
9. o-chloro-phenol indophenol	All stained.	All stained.

This table is in many respects interesting. The pink vital stain produced by neutral red is, of course, well known. But what is remarkable is that a rapid change-over occurs between Nos. 5 and 7. Above that point no colour is seen in the cells; below that point all the cells are coloured. In view of the fact that this change does not occur at the junction between the indigos and indophenols it might sound plausible to say that the reason why Nos. 1 and 5 did not stain was not a difference in permeability of the cells to the different dyes, but because they had been reduced by the cell interior, while Nos. 6 to 9 had not. Unfortunately, however, a glance at the table suffices to show that they are arranged in order of ascending, not descending, electrode potential. The extraordinary conclusion follows that the cells have been able to reduce those dyes most difficult to reduce, but have failed to alter the easier ones. Obviously another method is needed to test so curious a result. Happily in this case we possess the method of micro-injection.

B. Determination of the Internal pH by Micro-Injection.

The results obtained with the different types of egg are summarised in Table III.

Paracentrotus lividus—Unfertilised.

The first dye to be injected into these cells was neutral red. It permeated the cytoplasm and produced a distinct pink colour, corresponding to a pH less than 7.2. If some were allowed to escape into the drop at the same time as the injection, the pink egg could be well seen standing out against a background of yellow. If, however, the sea-water was the slightest degree con-

centrated, the dye was precipitated in the form of red needle-like crystals. In no case was cytolysis observed with this dye; the cells seemed to tolerate its presence exceedingly well.

Table III.

	Neutral red.	Brom-thymol blue.	Brom-cresol purple.	Methyl red.	Brom-phenol blue.	Result pH.
<i>Paracentrotus lividus</i> (unfertilised)	Pink	Yellow	Purple	Yellow	Violet-blue	6.6 \pm 0.1
<i>Paracentrotus lividus</i> (fertilised)	Pink	Yellow	Purple	—	—	6.6 \pm 0.1
<i>Echinocardium cordatum</i> (unfertilised)	—	Yellow	Purple	—	—	6.6 \pm 0.1
<i>Asterias glacialis</i> (unfertilised)	—	Yellow	Purple	—	—	6.6 \pm 0.1
<i>Asterias glacialis</i> (fertilised)	—	Yellow	Purple	—	—	6.6 \pm 0.1
<i>Ophiura lacertosa</i> (unfertilised)	—	Greenish-yellow	Purple	—	—	6.75 \pm 0.1
<i>Ascidia menula</i> (unfertilised)	—	Yellow	Purple	—	—	6.6 \pm 0.1
<i>Sabellaria alveolata</i> (unfertilised)	—	Yellow	Purple	—	—	6.6 \pm 0.1

The injection of this indicator, as also that of methylene blue, was found to be a matter of great difficulty because it coagulated the gelatinous envelope which surrounds the egg before fertilisation. If the pipette does not pierce the cell membrane the first time, the orifice usually becomes blocked by a plug of coagulated jelly deeply stained by the dye. Consequently our injections with this indicator are few and do not exceed 20–25 eggs, whereas with every other indicator the number is anything from 50 to 250.

The next indicator to be injected was brom-thymol blue. It was made yellow in the pipette corresponding to a pH of 6.7 or less and injected into a considerable number of eggs. In the drop it naturally turned blue as soon as it left the pipette, but in the egg it remained perfectly yellow. When the dye was made blue in the pipette and then injected the results were exactly the same. The pipette end could be seen in the cell and in its immediate neighbourhood the dye was blue, but wherever it came into contact with the protoplasm it took on a yellowish-green appearance corresponding to pH 6.4 to 6.8. When the pipette was withdrawn the whole egg looked yellowish-green, and if too much had been injected after about a minute cytolysis would take place, not explosively, but by a gentle streaming-out of the egg contents through holes in the membrane.

Brom-cresol purple was next investigated. The first attempt was with the

dye red in the pipette, about pH 6.0. On entering the cell it at once took on a very obvious purple colour, almost a deep mauve. This was very important as it fixed the cell as not being more acid than 6.4. Experiments were continued with the indicator both purple and yellow in the pipette. In every case the results were the same. The colour in the cell was mauve, and lasted usually from 30 to 50 seconds. Its definiteness and its transience are best expressed by calling it a "purple puff." The phenomenon was invariably seen, although an exceedingly large number of eggs were injected. If the egg was carefully observed at the moment of injection, the dye could be seen to change colour from yellow to purple immediately it left the orifice of the pipette, and then if attention was directed to the periphery of the cell or of the coloured part of the cell, it could be seen that after a certain time the edges of the purple area became green or greenish-yellow. Steadily this would spread until, as the last of the purple colour was disappearing, the whole cell would cytolysed completely. Experiment showed that this could be hastened artificially. If, immediately the injection was completed, the pipette was again brought into the cell and used as a micro-needle to tear the cell to pieces, it was seen that wherever injury had been produced there the purple would turn to green. The general rule that cytolysis is associated with the production of acid is well exemplified by the cytolysis of these eggs.

If these experiments were performed in ordinary sea water, it was often found on returning to the field of former exploits, that many, if not all, of the injected and cytolysed cells had again gone bright bluish-purple, although when last seen they had been greenish-yellow. The explanation of this was found when cells were placed in sea-water containing brom-cresol purple (purple, of course, because the pH was 8.4) and the cells then torn with a micro-needle. The injured parts at first went green, but if watched soon became purple. Then, if the same experiment was repeated in sea-water which had been brought to a pH of 6.0, it was seen that, although the cytolysed and torn cells went green, yet they never turned blue or purple afterwards. So that the subsequent change from yellowish-green to purple is one which must be put down to the alkalinity of the sea-water acting upon the dead organism. This point is of importance in view of other researches in this field mentioned in the general discussion.

Another exceedingly important point which emerges from these experiments with sea-water at pH 6.0 is that, although the eggs were allowed to stay in it for an hour and a half, yet they gave perfectly normal reactions at the end of that time. Excellent purple puffs with brom-cresol purple and a yellow colour

with bromthymol blue were uniformly observed. The conclusion must be that a change in the pH of the external environment of 2.4 units of pH does not produce changes in that of the internal environment greater than pH 0.2. But this subject is in itself a separate study and we did not pursue it further.

In the acid water the brom-cresol purple was yellow, so that after the injection the cell stood out purple against a yellow background. By the method of micro-test-tubes we were able to find that the mauve tint inside the cell corresponded to a pH of from 6.55 to 6.75, while the greenish-yellow tint of cytolysis was pH 5.75 or below.

To fix the pH of cytolysis further work was necessary. So methyl red was injected, and, although for reasons of solubility it had to be injected rather weaker than the other indicators, it nevertheless showed itself to be yellow in the cell and to turn pink when cytolysis came about, whether naturally or by injury with a micro-needle. Consequently, the pH of the uninjured cell must be above 6.0 and that of cytolysis at least as low as 5.0. Whether it was lower remained uncertain. Brom-phenol blue was then injected. In the pipette it was yellow, in the drop it was violet, but in the cell it was always deep violet-blue and did not change at all on cytolysis. The blue protoplasm was dragged about on a micro-needle and generally maltreated, but could never be got to show any other colour except violet-blue.

As the general conclusion, therefore, it seems clear that though below pH 5.0 the acidity of cytolysis is certainly above 4.0. Much further work would be needed to fix it exactly, but as this was not our problem, we proceeded to study the effect of fertilisation upon the internal pH of the cells.

Paracentrotus lividus—Fertilised.

As will be seen from Table III the colours resembled those of the unfertilised eggs in every way. Eggs were injected not only immediately after fertilisation but also when the fertilisation membrane was well developed, at the beginning, middle, and end of the first cleavage, and in the two and four cell stages. Throughout the later periods we were especially on the look-out for rhythmic alterations of pH which might have been associated with the many rhythmic changes known to occur at that time (*cf.*, Herlant, 7; Spaulding, 13; Fauré-Fremiet, 6). We never observed the smallest indication, however, of such rhythmic changes, and we believe that if any such changes exist they cannot exceed $\text{pH} \pm 0.20$.

The spindle and its associated structures could not be observed when indicators had been injected; they seemed to obscure it. We did not observe

any localisation of special colours, either here or at any other time in our work, and we conclude that the cell has a very uniform pH. The experiment with the acid sea-water, showing that the final alkaline change is due to the alkalinity of normal sea-water, we repeated on the fertilised eggs with the same results as before. The injection of the two-cell stage produced interesting results. One cell would be injected, would show purple, and then would cytolysed and go green—all this time its brother would remain unaffected and at the conclusion of the cycle could be injected in its turn.

Finally, we investigated the eight-cell stage. Here just the same results were found. The purple puff was always seen, and in one case seven out of the eight cells were injected in succession, each one showing the characteristic cycle.

The intercellular fluid of the late stages was also investigated. At the sixteen-cell stage brom-thymol blue showed blue if injected between the cells; which come apart, leaving an intervening space. The same result is obtained if injections are made into the blastocoele cavity. We consider that the liquid which fills it is certainly more alkaline than pH 7.3, but as this was a separate study, we did no further experiments upon it. It would be interesting to know how its pH changes with changes in the pH of the environment.

The general conclusion, therefore, is that as the unfertilised cells gave an internal pH of 6.6, so do the fertilised ones. In this egg fertilisation brings about no change in the internal pH greater than ± 0.2 .

Echinocardium Cordatum.

Of all the eggs which we investigated those of *Echinocardium* were the best, considered as material for micro-injection. They were absolutely transparent, and only suffer from the disadvantage that the animal itself refuses to live in captivity and has to be caught fresh every time its eggs are needed. Some unripe eggs were injected, in certain cases into the cytoplasm, in others into the nucleus. The nucleus in the latter type took on a blue colour, which it retained for a long time even after the cytoplasm had cytolysed and the egg gone green. The effect looked like a stain and we draw no conclusions from it. If the injection was made into the cytoplasm the purple puff was seen as usual.

Asterias Glacialis—Unfertilised.

The eggs of *Asterias* seemed to be more resistant than any eggs previously tried, so that the purple colour was decidedly more lasting.

Asterias Glacialis—Fertilised.

As Table III shows, fertilisation makes no difference at all. These very large eggs permitted the observation that in the immediate vicinity of the micro-pipette brom-thymol blue was blue, not yet having been changed, then in the outspreading ring there was a thin zone of greenish-yellow, and, finally, the dye in immediate contact with the protoplasm was quite yellow. Brom-cresol purple behaved as usual, and fertilised developing eggs in all stages up to that of four-cells gave the purple puff. This was specially well seen in one experiment where a very weak sperm suspension was used so that only 50 per cent. of the eggs were fertilised. In this way a direct comparison could be made, and on this one coverslip 190 unfertilised and 130 fertilised eggs were injected in succession. Not the slightest difference in colour was perceptible. Like the unfertilised ones, the fertilised ones were often very resistant to cytolysis and retained the purple colour for a considerable time.

If the dye was injected into the nucleus, of an unripe cell, for example, the nucleus turned bluish-purple and remained so till after the cytolysis of the cell. This might be taken to mean that the nucleus possessed a pH of rather under 6.5. If cells in process of dividing into two were injected, the dye ran at once through the isthmus and both became coloured. This happened even at a very constricted stage. Another important observation was made with the fertilised eggs of *Asterias*. If fertilisation is carried out with a rather concentrated sperm suspension and the eggs are afterwards left for a time in conditions of bad oxygenation, many abnormal forms make their appearance. Undivided eggs protrude bulbs of protoplasm within their fertilisation membrane, three-cell stages occur, and some eggs may break down into a number of separate spheres of protoplasm. Types of all these morphologically degenerating cells were injected with brom-cresol purple, and although a very large number (about 180) were examined, it was found that hardly any gave any variation in the internal pH . One or two extreme cases of spontaneous cytolysis showed a green colour at once when brom-cresol purple was injected into them, but the rest, whatever fantastic shapes their asphyxia had caused them to assume, always showed the purple colour. We consider that this observation is of considerable theoretical importance and we shall return to it in the general discussion.

Ophiura Lacertosa.

Whether because they are surrounded by a membrane of uncommon resistance, or for some other reason, the eggs of *Ophiura* certainly do not

cytolysed easily with pH indicators. They were the most resistant of all our eggs.

Table III shows that brom-thymol blue, blue or yellow in the pipette, immediately took on a green or yellowish-green colour in the cell interior. Although certainly not blue it appeared to indicate a slightly higher pH than that of the three preceding egg-cells, but not more so than 0.2 pH. So resistant were these eggs that the purple colour of brom-cresol purple frequently persisted for five minutes.

Ascidia Mentula.

When brom-thymol blue is injected through the coat of test cells and into the egg-cell itself a perfectly definite yellow colour is seen exactly similar to that observed in *Paracentrotus* or *Asterias*. When brom-cresol purple is injected the immediate purple puff is also seen as is also the subsequent turning to greenish-yellow on cytolysis. Cytolysis here is always internal cytolysis—the membrane never breaks—but all the same the green changes to bluish-purple, owing to the alkalinity of the drop. If ordinary *Ascidia* eggs are placed in a drop of sea-water coloured with brom-cresol purple they stand out in it like perfectly transparent golf balls, and normally never stain. But if they are injured by tearing with a micro-needle they go at first green and then quickly purple all over. The skin cells never stain.

Sabellaria Alveolata.

With this egg, as was pointed out in the section on technique, dark-ground illumination was used. In this way it was possible to see brom-thymol blue yellow in the cell from the instant it left the orifice of the micro-pipette. With the dark ground the cells stood out like yellow and white counters on a black ground, and as one injection after another was made, cell after cell would go yellow, although the colour in the micro-pipette was known to be blue.

Additional confirmation of our figure for the internal pH of these eggs was obtained thus. As soon as No. 7 was injected it went red. This demonstrates that the pH must be at least more acid than 8.5, since at that point the change from blue to red occurs with this indicator. This is important in view of previous work on the eggs of *Sabellaria* to which we shall refer in the discussions.

We may sum up this section, then, by saying that all the egg cells we studied have an internal pH of 6.6, or in its very near neighbourhood. The only exception is *Ophiura lacertosa*, which is nearer 6.75. This is a whole unit pH

more acid than *Amoeba*, which we found to have a pH of 7.6. We did not detect any localisation.

C. *Determination of the Internal rH by Micro-injection.*

The results are collected together in Table IV. Our experience with the *Amoeba* showed us that with solutions more than about 75 per cent. reduced, no colour could be seen under the microscope. The dyes put down as reduced may, therefore, only be so to the extent of 75 per cent. On the other hand, as we had no facilities for the preparation of dyes reduced to different stages for accurate comparison, it is possible that the dyes put down as remaining oxidised may in reality have been slightly reduced, say 10 or 20 per cent. Therefore, in calculating the results, we have taken the equilibrium in the cell as lying between 25 per cent. reduction of the last dye apparently unaffected, and 75 per cent. reduction of the first dye in the series to be apparently completely reduced. For *Ascidia mentula*, where No. 6 is partially reduced, we have taken the equilibrium as at 50 per cent. reduction of this dye.

Paracentrotus lividus—Unfertilised.

No. 1 when injected into a cell rapidly permeated the whole of the interior and showed no sign of fading. Even after half-an-hour the colour would still be as it was in the beginning, and when many eggs were on one coverslip, one would return from the hundredth to the first few that had been injected always to find them perfectly blue. Sea-water seems to precipitate this dye but does so in the form of exceedingly fine granules which neither block the pipette nor affect the contents. The actual dye which is injected into the cell is thus quite in solution. Cytolysis never seemed to occur.

Very similar was the behaviour of No. 2. As a rule when injected it spread all over the cell, but occasionally it would collect into a vacuole. Cytolysis was more frequent with this indicator than with No. 1, but when it did take place there was a delay of from one to five minutes. What was important, however, was that no reduction whatever was seen; the cells remained blue indefinitely with no fading.

No. 3, the indigotine tetrasulphonate, behaved in exactly the same way. It permeated the whole cytoplasm when it was injected and only the slightest difference could be seen between the colour at the end of half an hour and that at the very beginning. Cytolysis with this dye was rarer than with No. 2, and its effects in this respect more approached those of No. 1. In order to

Table IV.

	1.	2.	3.	3A.	4.	5.	6.	7.	8.	9.	Result. rH.
<i>Paracentrotus lividus</i> (unfertilised)	Oxidised	Oxidised	Oxidised	Oxidised	Oxidised	Oxidised	Reduced	Reduced	Reduced	Reduced	19.7-20.6
<i>Paracentrotus lividus</i> (fertilised)	Oxidised	Oxidised	Oxidised	—	—	Oxidised	Reduced	Reduced	—	Reduced	19.7-20.6
<i>Echinocardium cordatum</i> (unfertilised)	—	—	—	—	—	Oxidised	Reduced	Reduced	—	—	19.7-20.6
<i>Asterias glacialis</i> (unfertilised)	—	—	—	—	—	Oxidised	Reduced	Reduced	—	—	19.7-20.6
<i>Asterias glacialis</i> (fertilised)	—	—	—	—	—	Oxidised	Reduced	Reduced	—	—	19.7-20.6
<i>Ophiura lacerlosa</i> (unfertilised)	—	—	—	—	—	—	Oxidised	—	Reduced	—	21.3-21.7
<i>Ascidia mentula</i> (unfertilised)	—	—	—	—	—	Oxidised	Partially reduced.	Reduced	—	—	21.1
<i>Sabellaria alveolata</i> (unfertilised)	—	—	—	—	—	Oxidised	Oxidised	Indiv. differences	Reduced	—	21.7-22.2

prove that the retention of colour was a true absence of reduction and was not due to absorptive staining from small amounts of dye which had been allowed to escape into the hanging drop, some cells were placed in a drop containing a little of the indicator and were then torn, pricked, and mauled with a micro-needle. In spite of this they remained colourless indefinitely, showing that, since staining will not take place, even upon injury, it cannot mask reduction.

No. 3A, methylene blue, was quite unreduced in these cells, unlike the *Amœba*. Its injection is extraordinarily difficult, for it coagulates the gelatinous envelope, and the pipette point very soon has a mass of streamers of coagulated blue jelly attached to it. However, the few cells which we succeeded in injecting gave perfectly definite results. .*

No. 4, though a dye of an altogether different class, gave much the same results. There was not a suspicion of reduction. Cytolysis was infrequent, but even when it occurred the cytolysing fragments were blue, as was the eventual débris. This indicator has also a *pH* virage, but does not help us much for its change is rather outside the physiological area. It is blue down to *pH* 5.5, and red lower still.

The next indicator, No. 5, was also not reduced. Even after 20 minutes the colour was as strong as it had been immediately after the injection. This was in striking contrast to the *Amœba*, the results with which have already been described (10). In the *Amœba*, this indicator fades at once to a constant pale pink; here, it retains indefinitely its deep red colour. There were no cytolyses.

The change came at No. 6. When No. 6 was injected in the usual way the circumference of the outspreading circle of dye was markedly paler than that welling in from the pipette end, and by 20-30 seconds after the cell had been completely permeated by the dye, reduction would be complete. No colour at all would be left. Cytolysis occurred very often after two or three minutes, but it was always an internal cytolysis, the membrane not rupturing, but the protoplasm separating out into large globules. With this dye, and indeed with all the higher ones, it is necessary to avoid with care any possibility of staining effects masking reduction, for the cells stain readily with these indicators. A striking demonstration of the rapidity of the reduction of this dye is seen when one injects a row of 20 or 30 cells, one after another, and then looks back from time to time to see all stages of fading, the earlier ones completely colourless, the intermediate ones very pale blue, and the ones just injected deep blue. Regions where a little dye was split into the drop soon became colourless,

and since the field was packed tight with cells, the dye must have stained the cells in its immediate neighbourhood and then been reduced.

With No. 7 the reduction was also rapid and definite. Owing to the pH of the cell interior, this indicator went red as soon as it entered the cell, then immediately faded to pink, and a moment later was colourless. The time taken for the complete process was from 5–50 seconds. When a large number of eggs were being injected together on the same field, the impression was given that it was impossible to obtain a red egg, however much dye one injected, or however quickly one worked. But with staining the matter was easy, one had only to surround a cell with the dye and very soon it was a deep red. There were no cytolyses with this dye.

No. 8 was completely reduced in one or two seconds, and there were no cytolyses. An interesting observation was made on one egg which was injected once with indicator, which it reduced; it was injected a second time, it reduced the dye a second time; and, finally, some dye was injected into the drop in its immediate vicinity, whereupon it became stained. Its reducing power had been overcome. The reduction with this dye was more rapid than with any other of our indicators, even including No. 9.

No. 9 was certainly completely reduced in a very short time after injection, usually three to five seconds. In one case it was injected into the nucleus of an unripe cell and there completely reduced in the same time. When the reducing power was finally overcome and staining supervened, the cells took on a pink colour. As the dye entered the cell in the course of micro-injection a purple ring was clearly perceptible, but reduction was so quick that it rapidly disappeared. At pH 5.0 this indicator *in vitro* is frankly pink, at 6.0 reddish purple, at 6.5 purple, and at 6.75 purplish black. *A priori*, then, this would seem to confirm our figure of 6.6 for the internal pH , and would indicate that the cell was quite dead, with a low cytolysis pH by the time that staining had occurred. But we do not wish to lay stress on this consideration because the protein error of this indicator when used for determination of pH is not known.

One point should be mentioned here—the possibility that the fading of the colour in all these cases might be due, not to reduction of the dye, but to its diffusion out of the cell through an injured area caused by the entry of the pipette. Apart from the unlikelihood that this would constantly happen with some dyes and not at all with others, this possibility is negatived by the fact that when a little dye was allowed to escape into the drop from the micro-pipette, the cells which had become colourless were seen to stand out on a blue background, having no colour themselves.

Summing up, then, our first application of the series of indicators, we may say that we found Nos. 1, 2, 3, 4 and 5 entirely unreduced in the cell, but that Nos. 6, 7, 8 and 9 were at once reduced to the colourless leuco state.

Paracentrotus lividus—Fertilised.

As Table IV shows, fertilisation brought about no difference at all. All the dyes that were reduced before were reduced afterwards, and all those that remained oxidised before, remained oxidised afterwards. This rule held good for every stage in development up to that of eight cells, so that at no time was any rhythmic change in *rH* observable. It was interesting with Nos. 7, 8 and 9 to inject the four cells of a four-cell stage egg and observe them becoming colourless one after another.

Echinocardium Cordatum.

Only Nos. 5, 6 and 7 were injected into these eggs, and all gave the same results as before (see Table IV).

Asterias Glacialis—Unfertilised.

In a few cases with No. 5, which was not reduced, there seemed to be some fading of the dye when the cell cytolysed. This was the only occasion on which we saw anything that indicated an increase of reducing power as an accompaniment of cytolysis.

With No. 6, several incompletely mature eggs were selected and the dye injected into their nuclei only. There it was reduced in exactly the same time as if it had been injected into the cytoplasm, and as completely. It did not escape at all into the rest of the cell; the nuclear membrane seemed strong enough to contain it.

Asterias glacialis—Fertilised.

With No. 6, cells could easily be injected, allowed to reduce, reinjected, and allowed to reduce again. This was seen again and again on cells at all stages of their development, as far as the stage of eight cells.

An interesting observation was made with No. 7, namely, that if the dye was injected only under the fertilisation membrane it remained blue, but as soon as it was injected into the egg itself, it went pink. This points to the view that the *pH* of the liquid filling the space between the egg and the fertilisation membrane is above 8.0, and probably the same as the sea-water.

Ophiura Lacertosa.

Here the data are incomplete, for as is seen in Table IV. only two dyes were injected, Nos. 6 and 8. The results were not altogether easy to interpret. When No. 6 was injected, it was found that reduction was exceedingly doubtful. There were no cytolyses and after half-an-hour the cells still looked thoroughly blue. But there were certain definite changes which happened in the following order. On injection, the cell would go deep-blue all over, the spreading colour flowing round the large nucleus which remained uncoloured. In the first few minutes nothing happened, but then the colour faded somewhat in the cytoplasm and collected into definite granules or globules, which retained a blue colour. Meanwhile the nucleus became more and more coloured until at the end of ten minutes it was much the most coloured part of the cell. But nothing that could be called a definite reduction was seen. The progressive coloration of the nucleus recalls von Mollendorff's views on nuclear vital staining (9).

When No. 8 was injected the appearances somewhat resembled those of No. 6. The very important difference was that in the first minute the cytoplasm faded to a quite colourless state. Afterwards the nucleus coloured in just the same way as with No. 6, but still later it also faded. Our conclusion was that this dye is completely reduced by the cytoplasm and partially so by the nucleus. As far as the nucleus is concerned it is difficult to be sure, for according to von Mollendorff the nucleus never shows any colour until it is dead, in which case the reduction which we observed in it may be the effects of a *post-mortem* change.

We see therefore that; speaking generally, No. 6 is not reduced but No. 8 is. We were not able to inject No. 7, for our material was very limited.

Ascidia Mentula.

Great care was necessary with No. 5 because the cells stained very readily from the drop on the slightest injury. It was, however, quite clear that no reduction took place. Cytolysis was not seen.

No. 6 provided appearances whose interpretation was distinctly more difficult. In a typical good injection the dye spread only slowly throughout the cell, changing as it did do from purplish-blue to prussian blue. Shortly afterwards it faded to a faint grey, after which it did not change further. Under higher powers the faint grey had a definitely bluish tinge. The whole phenomenon closely resembled what we had seen previously with No. 5 in the case of the *Amœba*, and our conclusion was that here also was a case of partial

reduction. The prussian-blue effect—which was only seen with these eggs—received its explanation when we observed with the unstained cell that the test cells were coloured with a natural pigment of a faint yellow tint. In the injected cell the blue colour of the dye would be influenced by two layers of this yellow colour, one above and one below, which would have the effect of changing a purplish-blue to a prussian-blue, and which would act thus the more effectively the more the dye was reduced. There were no cytolyses with this dye. It was interesting to note that although on the least injury these cells would stain readily from the drop, yet even when they were deeply coloured in this way the test cells were always colourless, and could not be got to stain even when torn with a micro-needle.

When No. 7 was injected into these egg-cells, rapid and complete reduction was seen, taking from 50 to 90 seconds to attain completeness. Again, care was necessary over the staining, for with this indicator these cells stain spontaneously. The following curious phenomenon was seen several times. In an injected egg there would be an inner zone of deep red, where an injection had been made, and an outer periphery of colourless protoplasm which the dye had not reached. When the same cell was looked at two minutes later there would be an inner colourless zone where the dye had been reduced, while now the periphery would be deep red, owing to staining from dye allowed to escape into the drop near by. It was easy also to inject a cell and re-inject it after reduction, or to inject a little dye into the drop next to it, and watch it stain and reduce alternately, until it could reduce no more and became permanently coloured.

Sabellaria Alveolata.

Here the results showed strong evidence of an individual variability, with regard to No. 7. It was definitely reduced in some eggs and not at all in others; in all cases reduction was slow, and when it went to completion it took two or three minutes. The only conclusion could be that individual eggs behaved differently to this indicator, some of them possessing the power to reduce it and others not.

This section may be summed up by saying that the rH of these egg cells varies between the limits of 19 and 22; the detailed minor differences will be found in Table IV. The rH does not alter on fertilisation, there are no rhythmical changes in the early periods of development, and we could detect no localisation of reducing power in the cell.

GENERAL DISCUSSION.

The contradiction found between the results with vital staining and those with micro-injection we consider to be of the first importance. As we have seen, for *Paracentrotus lividus*, using the first method with the dyes 1, 2, 3, 4, 5, the cells remained uncoloured; while the dyes 6, 7, 8, 9 gave coloured cells, with the exception of a small proportion in No. 6. With micro-injection, on the other hand, introduction of small amounts of the dyes 1, 2, 3, 4, 5 resulted in permanently coloured cells, while upon the injection of similar amounts of Nos. 6, 7, 8 and 9 the dye in the cells rapidly faded to its leuco form. If a small quantity of one of the indicators 1, 2, 3, 4, 5 is spilt from the micro-pipette into the hanging drop it can be seen that the cells stand out colourless against the blue background, and as long as they remain uninjured no dye will enter them. With a trace of one of the indicators 6, 7, 8, 9 in the hanging drop, however, the adjacent cells soon become coloured at the periphery, and if the dye near them is small enough in quantity this coloration will disappear; if not, they will become completely blue. Their reducing power will have been overcome by excess of the dye and their rH altered; the same effect can be obtained by injections repeated sufficiently often.

It is important to note that the change over from reduction to no reduction in the cell does not occur at the same point in the series as the change from indophenols to indigotines. The explanation, therefore, cannot lie simply in the difference of chemical structures between the dyes. These results demonstrate, in fact, the service which the method of micro-injection can perform in overcoming two important difficulties inherent in vital staining. Firstly, substances to which the normal cell-wall is impermeable can be introduced into the cell with little or no injury; secondly, owing to the rapidity of the method and the minute amount of dye necessary, there is far less danger of an upsetting of the original equilibria and the establishing of unnatural ones. By the time that a cell has stained, it is probably far removed from its physiological normal.

The pH of certain of the egg cells used in this work had already been investigated, and it will be interesting to compare the results obtained. Vlès (15), using his method of cell-compression and crushing, found pH 5-6, usually nearer 6, for the eggs of *Paracentrotus lividus*. He also reported that on cytolysis the cell material became more alkaline. In the method of micro-compression a single cell, suspended in a drop of indicator, is rapidly squeezed by means of a special apparatus between a slide and a coverslip, and the pressure is as rapidly released. In this way a partial crushing of the cell is

brought about, and on release of the pressure, indicator is drawn into the cell. The cell is then rapidly washed free from indicator by a stream of some convenient liquid, only slightly buffered and near neutrality, and the colour in the cell is examined. In our experience the immediate result of injury or cytolysis has always been increased acidity, and it seems to us probable that this method of crushing, however carefully the crushing is reduced to a minimum, must result in a greater degree of mechanical injury to the cell than is involved in the entry of a micro-pipette. This in itself might account for the lower pH found by Vlès. But, further, in Vlès's technique about 60 seconds must elapse between crushing and freedom from dye, so that the cell cannot be examined at the moment when the indicator is entering it. We have found that the initial tint of the indicator when on the alkaline side very frequently gives place in less than a minute to a more acid tint, owing to the injurious chemical action of the dye in the cell.

Another method by which Vlès and his collaborators (16) obtained similar results was that of freezing a quantity of ripe eggs, grinding up the frozen mass, and taking the pH electrometrically at the thawing point. The objection here must be that the freezing of the eggs solid, with the consequent formation of ice crystals inside them, will probably lead to such far-reaching injury that thawing, even at low temperatures, will be followed by a rapid rise in acidity. It seems very likely to us that the lowness of Vlès's results, compared with our own, may be explained by such considerations as these.

The matter of increased alkalinity after cytolysis is far from clear however. We believe that the primary accompaniment of cytolysis is increased acidity; under certain conditions this might later be abolished by loss of CO_2 , but the only condition under which we have ever observed rise in pH following cytolysis was in the presence of sea-water (pH 8.4), when the possibility of the rise being due simply to impregnation with the sea-water cannot be overlooked. In sea-water at pH 6.0, which is less acid than the cytolysed cell, no cells containing brom-cresol purple could ever be seen to go purplish-blue after having once cytolysed and shown a green colour. Vlès does not seem to mention the medium in which his cytolysed cells were contained. With a mass of cells, as free as possible from sea-water, frozen, ground up, thawed, and left in the air for 30 minutes, he observed that the colour with brom-thymol blue changed gradually from yellow to greenish-yellow. In this case it is difficult to think of any reason other than loss of CO_2 to account for the rise. But we found no evidence of a post-cytolysis rise in pH when normal sea-water was absent.

The only other one of our cells whose internal pH had previously received attention was *Sabellaria alveolata*. Fauré-Fremiet (5), by staining with Nile blue and brilliant cresyl blue, had arrived at a pH of 12.0. Two years later, Reiss (11), using the method of micro-compression, obtained a value of less than pH 5.6, and pointed out that with cells which contain a large amount of lipoids, as do those of *Sabellaria*, Nile blue gives a pink colour owing to the solubility of the dye in its undissociated form in the lipoids. This colour may easily be mistaken for that of the dye ionised on its alkaline side. Our results with *Sabellaria* eggs are, of course, somewhat higher than those of Reiss.

Our results in general are in agreement with those of Schmidtman (19), who introduces minute granules of solid indicator into the cell on the end of a micro-needle, and observes their tint as they dissolve. He finds internal pH 's from 6.4 to 7.6, and these, so far, are our limits also. He observes the acidity of cytolysis, just as we do.

The increased reducing power observed during the cytolysis of *Amœba* was never found with the egg-cells, although it was carefully looked for.

The constancy of the rH values of these cells on fertilisation is probably important. It would be dangerous, in view of our present state of knowledge of the complicated systems in the cell, to draw too definite conclusions from this fact, but obviously a possible explanation of it would be that the same substances are involved in the oxidative processes before as after fertilisation, only in greater quantity after that event. This condition of affairs might be brought about by an increased permeability of intracellular membranes after union with the spermatozoon. The change would thus be quantitative rather than qualitative. This is exactly the conclusion which was arrived at by Warburg and Meyerhof (18) in their later work.

The constancy of the pH values under all conditions investigated is exceedingly striking. Neither moderately severe pathological changes, nor ontogenetic changes in the individual, nor phylogenetic changes as between one group of animals and another lead to any difference in the result. For the rH the constancy for the individual is similarly seen, but small variations between the different families exist. One is left with a picture of protoplasm as a sort of wax, out of which a diversity of morphological patterns are modelled: the wax itself remaining unchanged until an extremity of pathogenic influence may cause it to melt.

As regards the variations in the rH , results are as yet far too few to enable one to guess what degree of alteration is significant for the cell. The results which we have obtained on *Amœba* and egg-cells all lie between rH 17 and 22. Mans-

field Clark (3) has suggested, however, that certain anærobic organisms may have an rH slightly less than 0. We propose next to investigate this point, in the hope that the fixing of the physiological range of rH may lead to clearer ideas as to the extent of alteration likely to take place in the individual.

SUMMARY.

1. A study of the eggs of four echinoderms, one tunicate, and one polychæte worm has been made with the method of micro-injection. The conclusions rest on the appearances seen in 3,300 injected eggs.

2. The internal pH of all the eggs studied is in the close neighbourhood of 6.6.

3. The internal pH does not change on fertilisation, nor are any subsequent rhythmical changes to be seen. It is constant as far as the 16-cell stage.

4. The internal pH is not affected by local injury, nor by the morphological degenerations produced in asphyxia until cytolytic stages are reached.

5. On cytolysis the pH is lowered to a point below 5.0 and above 4.0.

6. The rH of the cells studied varies more than the pH , but within the limits 19 to 22.

7. The rH does not change on fertilisation and is constant as far as the 8-cell stage.

8. The rH does not appear to alter on cytolysis.

9. The method of vital staining and the method of micro-injection give diametrically opposite results : this is discussed in the text.

We thank Prof. Sir Frederick Hopkins, F.R.S., for his continual interest and help, and Professor Pérez for his kindness in placing at our disposal the resources of the Marine Biological Laboratory at Roscoff. Our gratitude is also due to the Government Grant Committee of the Royal Society and to the Council of the Senate of the University of Cambridge, for grants towards the expenses of this research. Finally, we warmly thank M. Marcel Prenant, Chef des Travaux at the Roscoff Laboratory, for his never-failing assistance and kindness.

REFERENCES.

- (1) Clark, 'Reprint No. 823, U.S. Publ. Health Reports' (1923).
- (2) Clark and Cohen, 'Reprint Nos. 826 and 834, U.S. Pub. Health Reports' (1923).
- (3) Clark, Cohen and Gibbs, 'Reprint Nos. 904 and 915, U.S. Pub. Health Reports' (1923).
- (4) Clark, Cohen and Sullivan, 'Reprint No. 848, U.S. Pub. Health Reports' (1923).
- (5) Fauré-Fremiet, 'Comptes Rendus Soc. Biol.,' vol. 88, p. 863 (1923).

- (6) Fauré-Fremiet, 'Comptes Rendus Soc. Biol.,' vol. 85, p. 1051 (1923).
 - (7) Herlant, 'Archives de Biol.,' p. 519 (1920).
 - (8) Meyerhof, 'Bioch. Zeit.,' vol. 35, pp. 246, 280, 316 (1911).
 - (9) von Mollendorff, 'Ergebnisse d. Physiol.,' vol. 18, p. 142 (1920).
 - (10) Needham and Needham, 'Roy. Soc. Proc.,' B, vol. 98, p. 259 (1925).
 - (11) Reiss, 'Archives de Physique Biologique,' vol. 4, p. 35 (1924).
 - (12) Shearer, 'Roy. Soc. Proc.,' B, vol. 93, p. 213 (1922).
 - (13) Spaulding, 'Biol. Bull.,' vol. 6, p. 224 (1924).
 - (14) Tschachotin, 'Comptes Rendus Soc. Biol.,' vol. 84, p. 85 (1921).
 - (15) Vlès, 'Archives de Physique Biologique,' vol. 4, p. 1 (1924).
 - (16) Vlès, Reiss and Vellinger, 'Comptes Rendus Acad. Sci.,' vol. 179, p. 349 (1924).
 - (17) Warburg, 'Zeit. Physiol. Chem.,' vol. 57, p. 1 (1908).
 - (18) Warburg and Meyerhof, 'Pflüger's Archiv,' vol. 148 (1912).
 - (19) Schmidtman, 'Bioch. Zeit.,' vol. 150, p. 253 (1924), and 'Klin. Woch.,' vol. 4, p. 759 (1925).
-

Chlorocruorin: A Pigment allied to Hæmoglobin.

By H. MUNRO FOX, Fellow of Gonville and Caius College, Cambridge, and
Balfour Student in the University.

(Communicated by Prof. J. Stanley Gardiner, F.R.S.—Received October 14, 1925.)

[PLATE 1.]

The name chlorocruorin was given by Ray Lankester (1867) to a pigment dissolved in the blood plasma of Sabellid, Serpulid and Chlorhæmid polychæte worms. Lankester showed that chlorocruorin is related to hæmoglobin, since he obtained a hæmochromogen from it. The pigment is burgundy-red when in concentrated solution, green when dilute. It will be shown below that while chlorocruorin is constructed on the same plan as hæmoglobin, the differences between the two are very much greater than the differences between specific hæmoglobins.

Chlorocruorin exists in an oxidised and a reduced state which are of almost the same colour. The reduced form in dilute solution is a slightly yellower green than the oxidised. The oxidised differs, however, spectroscopically from the reduced form in the same manner as oxyhæmoglobin differs from hæmoglobin. Oxychlorocruorin has two bands in the red-green part of the visible spectrum and reduced chlorocruorin one broader one. These bands are situated to the red of the corresponding hæmoglobin bands. Chlorocruorin like hæmoglobin, can act as a peroxidase (Lankester, 1870).

The present communication deals solely with the chemical aspects of chlorocruorin. The pigment will be treated from the biological standpoint in a following paper.

The work reported below was done in 1924 and 1925 partly at the Marine Biological Station, Roscoff, Brittany, and partly in the Zoological Laboratory, Cambridge. The ultra-violet spectrophotometry was carried out at Strasburg in Prof. Vlès' Laboratory of Biophysics. *Spirographis Spallanzanii* from Roscoff was studied, and *Branchiomma vesiculosum*, *Sabella pavonina*, *Myxicola infundibulum*, and *Pomatoceros triqueter* from Plymouth. At Cambridge living animals from Roscoff and from Plymouth were used, and at Strasburg *Spirographis* blood, sent from Roscoff.

I wish to thank Prof. J. Barcroft and Prof. Fred Vlès most sincerely for their valuable advice and help. I am grateful to the Directors of the Roscoff and Plymouth Marine Biological Stations for an abundant supply of material, and to Messrs. Keilin, Anson, and Mirsky for their continual interest. The expenses of the research were in part defrayed by Royal Society Grants.

Oxygen Affinity of Chlorocruorin.

In a previous communication (Fox, 1924) it was shown that oxychlorocruorin can be reduced both by a vacuum and by living tissues.* It may function, therefore, as a respiratory pigment, in the sense of absorbing a greater amount of oxygen than does water and giving up this oxygen to tissues.

The dissociation curve of oxychlorocruorin has not yet been made, but it can be shown qualitatively that chlorocruorin has a lesser affinity for oxygen than has hæmoglobin. In a mixed solution of oxychlorocruorin and oxyhæmoglobin the α -bands of each are sufficiently far apart to be seen separately (see p. 203 below). A mixture is made in which the intensities of the two α -bands are the same, and this mixture is reduced by sealing it up with a piece of living tissue. The oxychlorocruorin α -band is seen to vanish before the oxyhæmoglobin α -band. Further, if separate mixtures are made of polychæte blood containing oxychlorocruorin with (1) frog's blood, (2) human blood, and

* The inner layers of the mucous tube of *Myxicola* are coloured green by oxychlorocruorin. The latter, which is contained not only in the blood of Sabellids, but in much smaller amount in the coelomic fluid, probably exudes through the nephridia. The oxychlorocruorin in the mucus cannot be reduced by a vacuum nor by living tissue, even when the mucus is cut into thin slices. It can only be reduced by hydrosulphite when the mucus is ground in a mortar with this reducer. This suggests a colloidal protection effect, the oxychlorocruorin being protected by the mucin. Wurmser (1921) demonstrated a similar protective effect of colloids against the photo-oxidation of chlorophyll.

(3) earthworm's blood, the time interval between the disappearance of the oxychlorocruorin α -band and that of the oxyhæmoglobin is different in each case. It is shortest in the case of the frog, longer with human blood, and longest for the earthworm. This is the order of the oxygen affinities of the three hæmoglobins in question.

The oxygen affinity of chlorocruorin is affected in the same way as that of hæmoglobin by the H-ion concentration. This could not be deduced *a priori*, for Redfield and Hurd (1925) have shown that, while the oxygen affinity of the hæmocyanin of *Loligo* is decreased, that of *Limulus* hæmocyanin is increased by CO_2 .

In order to study the pH effect, oxychlorocruorin was first put into a series of buffers to find within what limits of pH it is stable. Below pH 7.0 and above pH 8.5 the intensity of the α -band fades, presumably owing to its changing into the derivative corresponding to hæmatin, although the first fading immediately below pH 7.0 must occur because at low pH values the chlorocruorin is incompletely saturated with oxygen. The wave-length of the band is uninfluenced by [H]. Equal concentrations of oxychlorocruorin (from *Spirographis* blood) were next put into buffers (1) of pH 7.0 and (2) of pH 8.5. These were exposed to varying pressures of oxygen in tonometers, and the pigment examined with a reversion spectrometer (Hartridge, 1912 and 1922) while inside the tonometer. The tonometer was first evacuated so that the oxychlorocruorin α -band vanished. Measured quantities of air were then successively admitted until the oxychlorocruorin α -band again acquired the same intensity as it has in atmospheric air. At pH 8.5 this was attained with 9 mm. oxygen pressure; at pH 7.0 between $\frac{2}{3}$ and 1 atmosphere of oxygen pressure was required. The temperature was 16°.

Since chlorocruorin has a lower oxygen affinity than hæmoglobin, and its dissociation curve is correspondingly shifted to the right, the question next arose as to whether chlorocruorin is fully saturated with oxygen at the atmospheric tension of the gas or whether a higher tension is required for the curve to reach its top and flatten out. Oxygen was bubbled through *Branchiomma* and *Sabella* bloods diluted with tap water, and it was found in both cases that the intensity of the α -band of oxychlorocruorin was increased by this procedure; the increase was more marked for *Branchiomma* than for *Sabella*. The experiment was then repeated with blood diluted with a buffer solution of pH 8.5, the upper pH limit at which chlorocruorin is stable. The result of bubbling oxygen through was the same as before. Thus at the highest possible pH, and consequently greatest oxygen affinity, oxychlorocruorin in these animals

is still unsaturated with oxygen at the atmospheric tension. Bubbling oxygen through diluted *Spirographis* blood again causes an increase in intensity of the α -band, but in this case the change is very slight. For *Myxicola*, however, oxygen causes no appreciable darkening of the band, so that the oxychlorocruorin in this case is saturated.

Just as there are different hæmoglobins, there exist then a series of specific chlorocruorins differing from one another in their affinities for oxygen. The four forms studied can be arranged in the following order of oxygen affinities, the first-named having the greatest and the last the smallest: *Myxicola*, *Spirographis*, *Sabella*, *Branchiomma*.

Total Oxygen Capacity of Spirographis Blood.

The quantity of oxygen bound by chlorocruorin in the blood of *Spirographis* was determined with a Barcroft (1914) micro-differential manometer.* This was possible since oxychlorocruorin, like oxyhæmoglobin, gives up its oxygen on the addition of ferrieyanide.

Each determination was made with 0.1 c.c. of blood. Undiluted *Spirographis* blood was obtained by inserting a capillary pipette into each of the two blood vessels at the base of the branchiæ. This situation was chosen owing to the absence of any coelomic cavity here. From eight individuals 0.1 c.c. of blood can thus be extracted. The value for the total oxygen capacity given below is higher than that announced in my preliminary communication (Fox, 1924). The reason is that previously the blood had been taken from the body of the worm, where, in spite of the greatest precautions, some admixture of coelomic fluid is unavoidable.

Determinations were first made of the oxygen capacity of my own blood. Seven estimations gave 20.4, 18.3, 19.0, 20.2, 19.3, 19.0, and 18.6 c.mm. O_2 at N.T.P. from 100 c.mm. blood. The average of these values is 19.3.

The results of five estimations of *Spirographis* blood were 8.1, 9.2, 9.1, 10.0 and 9.2 vol. per cent. at N.T.P., the average being 9.1. The blood thus binds 15 times the quantity of oxygen dissolved in sea water (barometer during experiments, 759 mm.).

It has been shown above that the oxychlorocruorin of *Spirographis* is not saturated with oxygen at the atmospheric partial pressure. Further determinations were accordingly made with *Spirographis* blood, this time saturated with oxygen (barometer, 760 mm.). Under these conditions the total oxygen

* The apparatus was calibrated by liberating in one bottle a known quantity of oxygen from H_2O_2 .

capacity was found, in three estimations, to be 10.6, 10.1 and 9.8 vol. per cent. at N.T.P., with an average of 10.2. This means that at atmospheric oxygen pressure *Spirographis* oxychlorocruorin is $10.2/9.1 = 90$ per cent. saturated.

For comparison, determinations were next made of the oxygen capacity of the blood of a polychæte containing hæmoglobin. *Arenicola* was chosen. It is possible here to extract 0.1 c.c. blood from one worm, and each determination was made with a single individual. The values obtained differed widely from one another, which means that the hæmoglobin content of the blood is very variable. Barcroft (1924) had already noted this in colorimetric estimations. The oxygen capacities of six individuals were found to be 8.2, 5.7, 8.7, 6.8, 7.4, and 6.8 c.mm. O₂ in 100 c.mm. blood. These values are seen to be lower than those for *Spirographis*.

The oxygen capacities recorded above for the bloods of *Spirographis* (9.1, vol. per cent.) and of *Arenicola* (5.7 to 8.7 vol. per cent.) are the highest known among invertebrates. The highest value for blood containing hæmocyanin is 4 to 5 vol. per cent. for *Octopus* (Winterstein, 1909; Dhéré, 1919).

Absorption Spectrum of Oxychlorocruorin.

It has been pointed out above that the absorption spectra of oxychlorocruorin and chlorocruorin resemble those of oxyhæmoglobin and hæmoglobin.

Fig. 1 shows the appearance of the spectrum of oxychlorocruorin in the visible region. The α -band is much more intense than the β , and both are

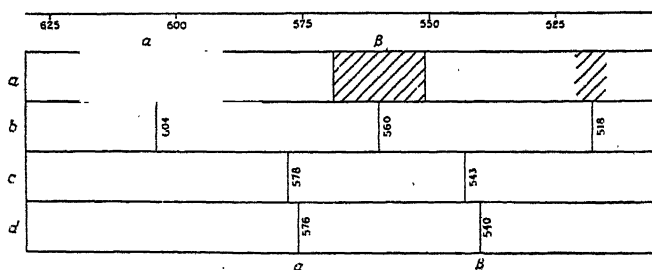


FIG. 1.—Absorption spectra of oxychlorocruorin and oxyhæmoglobin. (a) Oxychlorocruorin of *Spirographis*, general aspect of spectrum. (b) Axes of bands of (a). (c and d) Axes of bands of oxyhæmoglobin of the horse (c) and *Arenicola* (d). (a and b): spectrometer readings; (c and d) spectrophotometric determinations by Vlès (1923, p. 7).

shifted to the red of the corresponding oxyhæmoglobin bands. This shift is much greater than the differences between specific hæmoglobins (see fig. 1,

c and d). The axis of α is at $604 \mu\mu$, of β at 560 (spectrometer measurements*). Oxychlorocruorin has a third very light band at 518 . This has no representative in oxyhæmoglobin. Fig. 2 (b) gives a photograph of the spectrum (the band at 518 here appearing exaggerated).

Fig. 3 (a) gives the spectrophotometric curve in the visible of oxychlorocruorin. The measurements were made at atmospheric oxygen tension, so

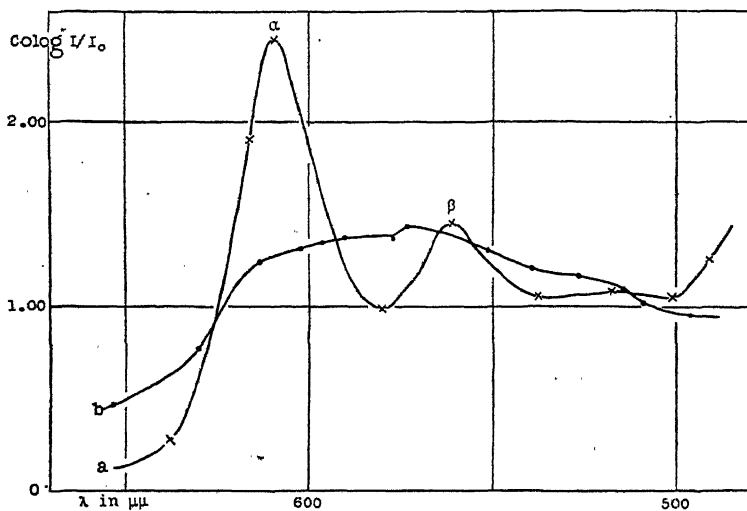


FIG. 3.—Spectrophotometric curves in the visible of (a) oxychlorocruorin and (b) chlorocruorin of *Spirographis*. The oxychlorocruorin solution is saturated with air. After the oxychlorocruorin had been determined it was reduced in the trough with hydro-sulphite so that the molecular concentrations are the same for the two curves.

The values from which these curves are drawn are given in the Appendix (p. 217).

that there is a 10 per cent. admixture of reduced chlorocruorin. The absorption ratio α/β is seen to be 1.69, as compared with 1.04 for hæmoglobin of the horse, 0.95 of *Arenicola*, and 0.88 of *Marphysa* (Vlès, 1923).

In the ultra-violet, oxyhæmoglobin has an intense band (γ) at 416 (see (Vlès, 1921, fig. 2). The absorption ratio α/γ is 0.08 only. There is a smaller band γ' at 343 (absorption ratio $\alpha/\gamma' = 0.34$). Bands γ and γ' are due to the pyrrol nucleus. Finally, there is a band, ϕ , at 275 , due to the protein (globin).

The photograph fig. 2 (c) (Plate 1), shows the γ -band of oxychlorocruorin.

* The wave-lengths of the axes of bands determined with the spectrophotometer (fig. 3 and Table I), the ordinary spectrometer (fig. 1), and Hartridge's reversion spectrometer (Table II) differ from one another. This is to be expected from the differences in methods used (Hartridge, 1913, and Vlès, 1921, p. 15).

The spectrophotometric* curves of fig. 4 show the γ -, γ' -, and ϕ -bands of oxy-chlorocruorin saturated (a) with oxygen and (b) with air. Solution (b) consequently contains a 10 per cent. admixture of reduced chlorocruorin. The absorption ratio γ/γ' appears to be 1.5 from curve (a) or 2.3 from curve (b).

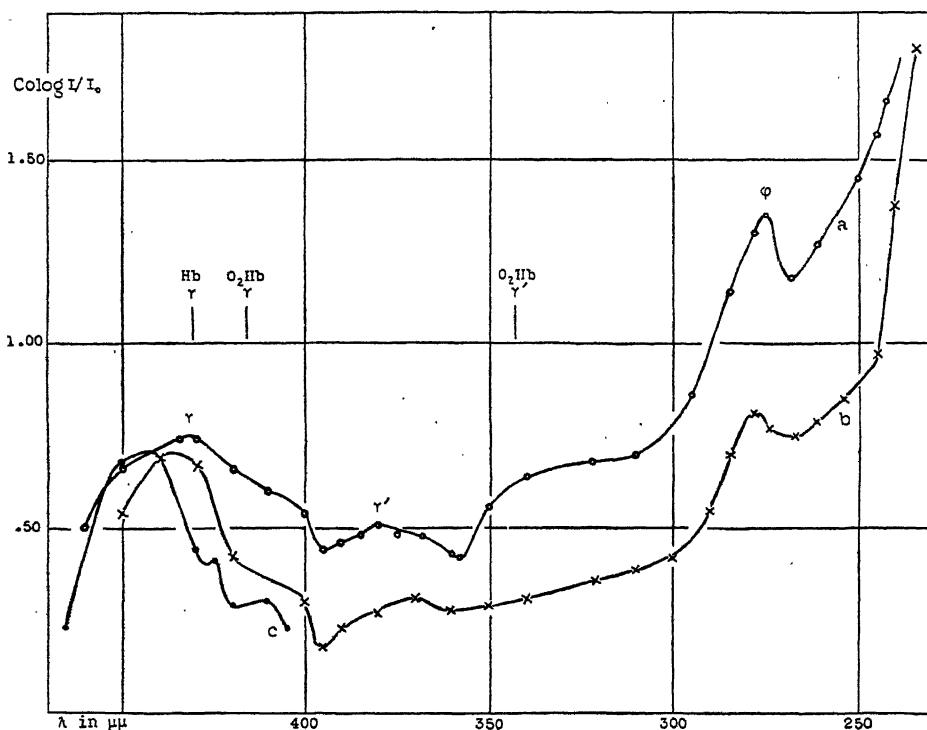


FIG. 4.—Spectrophotometric curves in the ultra-violet of oxy- and reduced chlorocruorin. Diluted *Spirographis* blood (a) saturated with oxygen, (b) saturated with air, (c) reduced with hydrosulphite. The concentrations are identical in the three cases. The wave-lengths of the axes of the oxyhæmoglobin (O_2Hb) and hæmoglobin (Hb) γ - and γ' -bands are inserted for comparison.

The values from which these curves are drawn are given in the Appendix (p. 218).

Although these figures have here no absolute value, owing to the general absorption by other colloids of the diluted whole blood, yet the ratio is evidently lower than that for oxyhæmoglobin. In the latter case the γ -band is considerably more intense relatively to the γ' , the ratio being 4.1 (Vlès, 1921, p. 9). Table I compares the positions of the axes with those of the corresponding hæmoglobin bands. As in the case of α and β , γ and γ' are shifted to the red in oxychlorocruorin as compared with oxyhæmoglobin.

* For the ultra-violet spectrophotometry the technique of Prof. Vlès (1925) was used.

Table I.—Spectrophotometric axes of the bands of (I) oxyhæmoglobin of the horse (Vlès, 1921, p. 7), and (II) oxychlorocruorin of *Spirographis*.

Designation of Band	α	β	—	γ	γ'	ϕ
I	578	543	—	41(6)*	34(3)	27(5)
II	609	561	517	43(2)	37(9)	27(5)

* Figures in brackets are uncertain.

Specific Chlorocruorins.

It has been shown above that there are specific chlorocruorins with varying oxygen affinities. Now there exists a series of hæmoglobins spectroscopically different from one another (Sorby, 1876 ; Vlès, 1923 ; Barcroft, 1924), and in just the same way the specific oxychlorocruorins differ in the wave-length of the α -band.* Table II gives details of this. The α -band in *Sabella* differs

Table II.—Positions of α -bands of oxy- and CO-chlorocruorins measured in Ångstrom units with the Hartridge reversion spectrometer.

Animal	Position of α -band		Span, or Difference between Oxy- and Carboxy- α -band
	Oxy-chlorocruorin	Carboxy-chlorocruorin	
<i>Sabella</i>	6059	6011	48
<i>Myxicola</i>	6056	6027	29
<i>Spirographis</i>	6048	6016	32
<i>Branchiomma</i>	6029	5987	42
<i>Pomatoceros</i>	6025	6008	17
	Oxy-hæmoglobin	Carboxy-hæmoglobin	
Man	5763	5700	63
Planorbis	5745	5695	50

* MacMunn (1885) described specific chlorocruorins of Serpulids, differing in colour, some red, some brown, others green. In my experience chlorocruorin is always of the same colour, red concentrated, green dilute, although the tint seems to vary slightly from one species to another. The diluted blood of *Sabella*, for instance, is of a more yellowish green than that of *Spirographis*. This, however, may be due to an additional pigment. MacMunn's assertion can perhaps be explained by such additional pigments in the tissues of Serpulids. When *Pomatoceros* is cut up in fresh water a pink solution is formed. In addition to oxychlorocruorin there is present a substance with two bands at 539 and 499 (reversion spectrometer). Sodium hydrosulphite and living tissues both reduce this

from that in *Pomatoceros* by 34 Ångstrom units. This is a greater variation than that between two of the most divergent hæmoglobins, human and *Planorbis*, which differ by 18 Å only.

Absorption Spectrum of Reduced Chlorocruorin.

Fig. 5 gives the absorption spectrum of chlorocruorin (reduced with sodium hydrosulphite) in the visible region. The Stokes' bands of *Arenicola* and

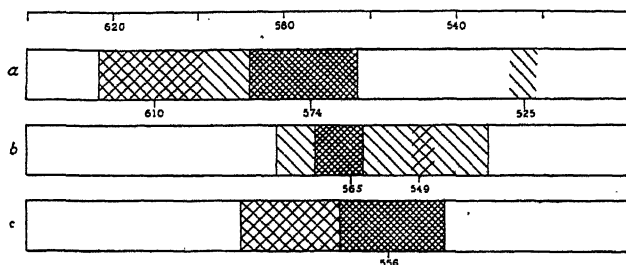


FIG. 5.—Absorption spectra of reduced chlorocruorin and hæmoglobin. (a) Chlorocruorin of *Spirographis*. (b) Hæmoglobin of *Arenicola*. (c) Mammalian hæmoglobin. (b and c from Vlès, 1923, fig. 5.) All reduced with hydrosulphite. The numbers give the axes of the bands, in (a) determined with the spectrometer, in (b) and (c) spectrophotometric measurements by Vlès (1923, p. 17, and 1921, p. 12).

mammalian hæmoglobins are inserted for comparison. The chlorocruorin band is again shifted to the red. It has a darker axis at 574 and a lighter one at 610.* In addition, there is a faint band at 525. Fig. 2 (d) gives a photograph of these bands (the intensity of the band at 525 appearing exaggerated).

Fig. 3 (b) gives the spectrophotometric curve of reduced chlorocruorin in the visible. It shows the absorption relative to that of oxychlorocruorin and explains the appearances seen in figs. 5 (a) and 2 (d). There is, in reality, a main summit at 572 with a buttress on either side. The Stokes' band of mammalian hæmoglobin has a simple summit (Vlès, 1921, fig. 3) and that of *Arenicola* hæmoglobin a double apex (Vlès, 1923, fig. 4).

Reduced hæmoglobin has a γ -band situated to the red of that of oxyhæmoglobin. Its axis is at 431 (Henri et Wurmser, 1912), while that of oxyhæmoglobin is at 416. Similarly, the γ -band of reduced chlorocruorin is to the red

pink pigment to a colourless substance which permits the green colour of chlorocruorin to appear in the solution. The reduced substance re-oxidises in air. This pink pigment is at present under investigation. The normal green of chlorocruorin, however, can be seen under the microscope in the blood-vessels of *Pomatoceros*.

* Reduced with ammonium sulphide the main band has a single axis at 589. Vlès (1923, p. 17) has shown that the spectrum of *Arenicolan* hæmoglobin, too, varies with the reducing agent employed. Mammalian hæmoglobin, on the other hand, is invariable.

of that of oxychlorocruorin. This band is seen in the photograph, fig. 2 (*d* and *e*). The spectrophotometric curve is given in fig. 4 (*c*). The axis of the γ -band of reduced chlorocruorin is at 442 as compared with 432 for oxychlorocruorin. The absorption is the same for both forms. Fig. 2 (*e*) shows another band on the side of γ away from the visible. This is seen at 425 on the curve (fig. 4 (*c*)).

The γ' - and ϕ -bands of reduced chlorocruorin could not be studied owing to the general absorption in this region by the colloidal hydrosulphite.

Derivatives of Chlorocruorin.

Not only are chlorocruorin and hæmoglobin spectroscopically similar, but a series of derivates can be prepared from chlorocruorin corresponding to those obtained from hæmoglobin.

Like hæmoglobin, chlorocruorin has a greater affinity for CO than for oxygen. CO-chlorocruorin has a spectrum resembling that of oxychlorocruorin with the α - and β -bands shifted towards the blue. As in the case of CO-hæmoglobin, the CO-chlorocruorin α -band is fainter and broader than the oxychlorocruorin α -band. The amount of shift of the α -band when CO displaces O₂ is called by Barcroft the "span." Table II gives the spans of several specific chlorocruorins, determined with the reversion spectrometer. The spans of human and *Planorbis* hæmoglobins are inserted for comparison.* They are greater than the chlorocruorin spans. Light dissociates CO-hæmoglobin. It has a similar effect on CO-chlorocruorin but to a more pronounced degree.

Metachlorocruorin of a brown-green colour is formed on the addition of potassium ferricyanide to oxychlorocruorin. The spectrum shows three bands at 604, 569 and 518 (spectrometer readings). There is no band in the red. The band at 569 is more intense than that at 604. On the addition of sodium carbonate the band at 604 becomes more intense than that at 569, which moves to 564. On acidifying again there is no further change, so that the alteration on addition of sodium carbonate may have been due to a change in turbidity, not to the appearance of an alkaline metachlorocruorin. Vlès (1923, p. 19) has shown that the methæmoglobin of *Arenicola*, too, is aberrant, but in a different sense.

Weak acids convert oxychlorocruorin into acid *chlorocruorohæmatin*. When *Spirographis* blood is diluted with sea water the addition of citric acid gives a heavy precipitate, but when diluted with distilled water a clear solution is

* The values given here differ from those of Anson, Barcroft, Mirsky and Oinuma (1924, p. 62), using the same instrument. The values of all spans given by these authors require modification.

obtained. It is reddish brown. The absorption spectrum shows a well-defined band with its axis at 562. In addition, there are less defined bands at 670 and 504.* On making the solution alkaline, it takes on a greenish-brown colour. Its spectrum shows two well-defined bands at 630 and 588. In addition, there are less defined bands at 683 and 475.

It is thus apparent that the spectra of acid and alkaline chlorocruorohæmatin have a character different from those of acid and alkaline hæmatin. This being so it is remarkable to find that chlorocruorohæmatin in ether plus glacial acetic acid has a spectrum quite analogous to that of hæmatin in the same solvent. The method employed was to drop *Spirographis* or human blood into a mixture of 2 parts of ether to 1 part of glacial acetic acid. In each case a four-banded spectrum is obtained (see fig. 6), the bands of chlorocruorohæmatin being to the red of those of hæmatin. In chloro-

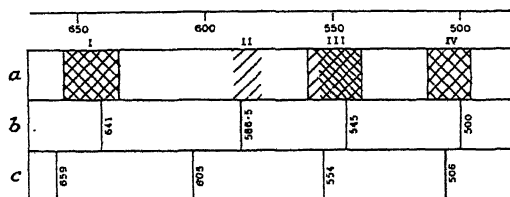


FIG. 6.—Absorption spectra of hæmatin and chlorocruorohæmatin prepared with ether and acetic acid. (a) General aspect of the hæmatin spectrum, determined with the ordinary spectrometer. (b) and (c) Wave-lengths of the axes of the bands of hæmatin (b) and chlorocruorohæmatin (c), determined with the reversion spectrometer.

cruorohæmatin band I is darker than III, in hæmatin the reverse is the case. Band II is very light in both.

Spirographis blood dried on a microscope slide and treated with the usual procedure for preparing hæmin gives crystals which resemble the latter.

While, however, the derivatives corresponding to methæmoglobin and hæmatin in watery solution do not closely resemble these substances, a typical hæmochromogen can be prepared from chlorocruorin. The method used was either to reduce the oxychlorocruorin with hydrosulphite and then add alkali, or to add acid, then alkali, and lastly to reduce. The spectrum of this *chlorocruorochromogen* is shown in fig. 7. The bands are to the red of those of hæmochromogen prepared from hæmoglobin, but the difference is less than that between oxychlorocruorin and chlorocruorin compared with

* This implies a correction to the absorption spectrum given in my preliminary communication. The reason is that previously the clear solution in distilled water had not been obtained.

oxyhæmoglobin and hæmoglobin. The α -band of chlorocruorochromogen is asymmetrical and less intense than that of hæmochromogen. Further, unlike hæmochromogen, the bands do not immediately attain their definitive positions. There is an initial stage of chlorocruorochromogen which only gradually changes into the final state (see fig. 7).

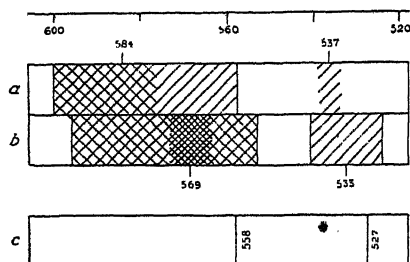


FIG. 7.—Absorption spectra of chlorocruorochromogen and hæmochromogen. (a) Initial stage, and (b) final stage of chlorocruorochromogen. (c) Axes of bands of globin-hæmochromogen. Spectrometer measurements.

As in the case of hæmochromogen, the addition of KCN to chlorocruorochromogen causes the bands to become less intense and to move towards the red. Their axes move from 569 and 533 to 573 and 538.

Anson, Barcroft, Mirsky and Oinuma (1925, p. 75) have demonstrated with Hartridge's reversion spectrometer that the hæmochromogens prepared from the hæmoglobins of various vertebrates and insect larvæ all have the axis of the α -band at the same wave-length. This does not necessarily mean that all of these hæmochromogens are identical, for Vlès (1923, p. 20) has shown spectrophotometrically that the hæmochromogen of *Arenicola* absorbs less than that of the horse. It was of interest, however, to know whether the specific chlorocruorins, too, would give chlorocruorochromogens all alike as regards the wave-length of the α -band. Such was found to be the case. Preparations were made from the bloods of *Branchiomma*, *Sabella* and *Myxicola*. For all three forms the position of the axis of the α -band, measured with the reversion spectrometer, was the same. The wave-length was: (a) in the initial stage, immediately after preparation, 5810 Å, and (b) in the final state, measured next morning, 5657 Å.

The Protein and the Coloured Group of the Chlorocruorin Molecule.

Protein precipitants were used to test for the presence of a protein in chlorocruorin. Chloracetic acid brings down a precipitate in *Spirographis* blood. On centrifuging it is seen that the supernatant liquid is colourless.

The following procedure was adopted to confirm the presence of a protein. It allowed of a colour reaction being used, which is of course impossible in presence of the coloured portion of the chlorocruorin molecule. Schulz's classical method of splitting hæmoglobin into globin and the coloured group, consists in first converting the hæmoglobin into acid hæmatin and then adding ether and a little alcohol. The hæmatin then all passes over into the ether while the globin remains in the watery phase. *Spirographis* blood was treated in this way, chlorocruorohæmatin being first prepared from the oxychlorocruorin with a dilute acid. When ether was added, the coloured group passed over into the latter. The acid watery phase, which was colourless, could now be tested for a protein, and was found, in fact, to contain one. The biuret test was positive and tungstic acid gave a precipitate.

Chlorocruorin appears thus to be a conjugated protein, although it may be objected that the tests merely demonstrate some other protein in the blood. The existence of specifically different chlorocruorins argues, however, in favour of a protein rather than some simpler constituent of the chlorocruorin molecule.

The next question to be answered was, What is the relationship of the coloured group in the chlorocruorin molecule to that in hæmoglobin?

Bartin-Sans and Moitessier (1893) showed that the hæmochromogens are a family of substances formed when egg-albumin, amines or ammonia are added to reduced hæmatin, and that while all have the typical hæmochromogen spectrum the wave-lengths of the bands vary a little in each case. Anson and Mirsky (1925, p. 50) have taken up the question again, and, using Hartidge's reversion spectrometer, have fixed the axis of the α -band for a series of artificial hæmochromogens containing different nitrogen compounds attached to the hæmatin nucleus. One of their hæmochromogens was formed by adding globin to hæmin dissolved in NaOH and reduced. This gave an α -band in an identical position with that of ordinary hæmochromogen prepared directly from hæmoglobin. Hence, they conclude, ordinary hæmochromogen contains globin.*

Now, there exist several natural pigments other than hæmoglobin which belong to the same hæmatin family. Such are (1) cytochrome, a mixture of hæmochromogens present in most animal and plant cells (Keilin, 1925);

* Anson and Mirsky consider that hæmoglobin is a polymer of hæmochromogen. If this be so, the initial stage of chlorocruorochromogen may consist of, say, two molecules of the final stage, while chlorocruorin would consist of four molecules of the latter.

(2) helicorubin, a hæmochromogen in the gut of snails; and (3) actinohæmatin in the anemone *Actinia equina*.

Anson and Mirsky (1925, p. 161) attacked the problem next as to whether these different pigments have the same or a different hæmatin nucleus (called by them hæm). They did not examine the hæmatins directly, but from each pigment prepared an artificial ammonia-hæmochromogen. This procedure was adopted because the wave-length of the hæmochromogen α -band can be very accurately fixed (to within 2 Å) with Hartridge's reversion spectrometer. It was found that the wave-length was identical for the ammonia-hæmochromogens of (1) various hæmoglobins, (2) a portion of the cytochrome complex, (3) helicorubin, and (4) actinohæmatin.

Hence these authors conclude that the coloured group (hæmatin, or, as they term it, hæm) of all these pigments is identical. It should be pointed out, however, that while the fact just recorded shows a very close similarity between the hæmatins in question, it does not imply identity. In order to demonstrate this, the spectrophotometric curves must be prepared and the ratios between the absorption constants at different wave-lengths must be shown to be identical for each substance.

Chlorocruorin was next examined with the object of seeing whether its hæmatin nucleus is similar to those of the pigments mentioned above. In view of the fact that the various pigments of the hæmatin family studied by Anson and Mirsky turned out to have hæmatin nuclei either identical or very similar to one another, it might reasonably have been expected that the coloured group of the chlorocruorin molecule would fall in the same category. It was therefore surprising to discover that the hæmatin nucleus of chlorocruorin is very different.

The procedure was as follows: Ammonia-chlorocruorochromogen was prepared from *Spirographis* blood and the wave-length of its α -band compared on Hartridge's reversion spectrometer with that of ammonia-hæmochromogen. The wave-lengths of the two turned out to be far from alike. The α -band of ammonia-chlorocruorochromogen is 20 Å to the red of that of ordinary chlorocruorochromogen prepared directly from chlorocruorin. On the other hand, the α -band of ammonia-hæmochromogen is 27 Å to the blue of ordinary hæmochromogen made from hæmoglobin. The α -bands of ordinary chlorocruorochromogen and ordinary hæmochromogen are themselves 85 Å apart.*

* Chlorocruorochromogen 5657 Å, hæmochromogen from hæmoglobin 5572 Å (reversion spectrometer measurements).

Thus the ammonia-hæmochromogens are not at all similar, and hence the hæmatins of chlorocruorin and of hæmoglobin are unlike.

As to the mode of preparation, ammonia-hæmochromogen can easily be made by adding ammonia to ordinary globin-hæmochromogen. Ammonia has a great affinity for hæmatin and it displaces the globin. It was found, however, that this cannot be done with chlorocruorochromogen. Its protein has too great an affinity for its hæmatin group to be displaced by ammonia. Recourse must be had to another procedure. Chlorocruorin is split in Schulz's way. Ammonia is then added to the ether phase and the hæmatin passes over into the ammonia. A reducer (hydrosulphite) is added, ammonia-chlorocruorochromogen being thus formed. Naturally, the ammonia-chlorocruorochromogen was compared with ammonia-hæmochromogen prepared from hæmoglobin by an identical procedure. Ammonia-chlorocruorochromogen is a greenish-yellow solution, while ammonia-hæmochromogen is pink.

Concerning the protein part of the chlorocruorin molecule, all that can be said at present is that relatively to ammonia it has a greater affinity for its coloured group than has globin for hæmatin, since ammonia displaces globin from globin-hæmochromogen but not the protein from chlorocruorochromogen. The nitrogen compound of heliocorubin has a similar high affinity for its hæmatin group (Anson and Mirsky, 1925, p. 221). Further, the protein of chlorocruorin can be made to combine with the hæmatin of hæmoglobin. To bring this about chlorocruorin was split in Schulz's way and the acid watery portion containing the protein made alkaline. Hæmatin was then prepared by dissolving hæmin crystals in NaOH. Hydrosulphite was added and the product mixed with the protein from chlorocruorin. This hybrid has a typical hæmochromogen spectrum with its α -band within 3 Å of that of ordinary globin-hæmochromogen. The chlorocruorin protein has a good affinity for ordinary hæmatin, for it was necessary to add to the latter a smaller quantity of this protein to form a hæmochromogen than of any other protein except globin.

Constitution of the Coloured Group of the Chlorocruorin Molecule.

The hæmatin group of chlorocruorin being different from that of hæmoglobin, etc., the next question asked was whether it is an iron compound or perhaps contains some other metal. The test for masked iron, however, was positive.

The iron is at present being estimated quantitatively in order to establish

whether or not oxychlorocruorin resembles oxyhæmoglobin in having two atoms of oxygen united to one of iron.

Since the coloured group of the chlorocruorin molecule is an iron compound, the difference between chlorocruorohæmatin and the hæmatin of hæmoglobin must be due not to the metal but to the porphyrin with which the metal is united. In my previous publication (1924) it was left undecided whether or not chlorocruoroporphyrin is identical with hæmatoporphyrin. The method employed did not give sufficiently constant results for either product. Since, then, other methods have been adopted which show that, while chlorocruoroporphyrin and hæmatoporphyrin have the same patterns of spectra, the bands of the former are situated considerably to the red of those of the latter. The porphyrins, then, are different from one another. This means that, whereas both ordinary hæmatin and chlorocruorohæmatin contain iron, they differ in being built up from different groups containing pyrrol nuclei.

The method used in the preparation of the porphyrins was the following:—Blood of *Spirographis* and human blood were each dropped into concentrated H_2SO_4 kept cool under the tap. In both cases porphyrins were obtained having a broad unsymmetrical band in the green and a narrower symmetrical band in the red. The wave-lengths of the bands were measured six hours after

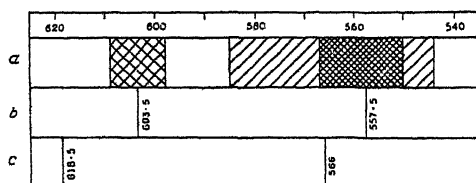


FIG. 8.—Absorption spectra of porphyrins prepared with concentrated H_2SO_4 . (a)

General aspect of the hæmatoporphyrin spectrum, determined with the ordinary spectrometer. (b) and (c) Wave-lengths of axes of bands of hæmatoporphyrin (b) and chlorocruoroporphyrin (c), measured with the reversion spectrometer.

preparation, since the positions are unsteady at first. The red band is more intense relative to the green band in chlorocruoroporphyrin than it is in hæmatoporphyrin. Fig. 8 gives the axes of the bands in both cases.

Hæmatoporphyrin is fluorescent (Dhéré and Sobolewski, 1916). Chlorocruoroporphyrin exhibits the same phenomenon.

Conclusion.

The hæmatin nucleus found in hæmoglobin, heliocorubin and actinohæmatin has a very wide distribution both in animals and plants, since it is also a constituent of cytochrome. Chlorocruorin stands apart in having a peculiar pyrrol

group, which is the more remarkable since there is a much greater similarity between the chemical structure of hæmoglobin and that of chlorocruorin than there is between hæmoglobin and any of the other pigments mentioned. Chlorocruorin, with its peculiar hæmatin, has a most restricted zoological distribution. It is found only in certain families of polychæte worms, while other families of these annelids have hæmoglobin. Now, the sporadic occurrence of hæmoglobin and related pigments in the animal kingdom may perhaps be accounted for by a common development with the almost omnipresent cytochrome. If this be the case, however, what is the origin of chlorocruorin? This is one of the principal problems connected with this substance which still remain to be solved.

Summary.

1. Chlorocruorin is a pigment dissolved in the blood plasma of certain polychæte worms. It is red in concentrated, green in dilute, solution.

2. Chlorocruorin exists in an oxidised and a reduced form, having absorption spectra analogous to those of oxy- and reduced hæmoglobin. The oxidised form can be reduced (a) by a vacuum and (b) by living tissue, and then reoxidised by air.

3. Chlorocruorin has a lesser affinity for oxygen than has hæmoglobin.

4. The oxygen affinity of chlorocruorin is increased by an increase in *pH*.

5. There is a series of specific chlorocruorins differing in oxygen affinities. The following are arranged in order of oxygen affinity from highest to lowest: *Myxicola*, *Spirographis*, *Sabella*, *Branchiomma*.

6. The chlorocruorins of *Branchiomma*, *Sabella* and *Spirographis* are unsaturated with oxygen when this gas is at the normal atmospheric pressure, while that of *Myxicola* is saturated.

7. The total oxygen capacity of the blood of *Spirographis* is (a) in contact with air, 9.1 vol. per cent.; (b) in oxygen, 10.2 vol. per cent. *Spirographis* oxychlorocruorin is thus 90 per cent. saturated at atmospheric oxygen pressure.

8. The absorption bands of oxychlorocruorin and reduced chlorocruorin resemble those of oxy- and reduced hæmoglobin shifted towards the red end of the spectrum.

9. In addition to this shift, the principal difference in the oxychlorocruorin spectrum compared with that of oxyhæmoglobin consists (1) in the high value of the absorption ratio α/β and the low value of γ/γ' , and (2) in the presence of an extra small band in the visible region between β and γ .

10. The specific oxychlorocruorins differ in the wave-length of the α -band axis.

11. The Stokes' band of chlorocruorin (reduced with hydrosulphite) has a summit with a buttress on either side, differing thus from the simple summit of the mammalian and the double apex of the Arenicolan hæmoglobin band. In reduced chlorocruorin the γ -band is shifted to the red of its position in oxychlorocruorin. (*Cf.* the same shift in reduced compared with oxy-hæmoglobin.)

12. Chlorocruorin has a greater affinity for CO than for oxygen. The spans of different chlorocruorins vary, but are always smaller than those of hæmoglobins. Light has a greater effect in dissociating CO-chlorocruorin than CO-hæmoglobin.

13. The spectrum of metachlorocruorin differs considerably from that of methæmoglobin.

14. Chlorocruorohæmatin in both acid and alkaline watery solution shows spectra unlike those of acid and alkaline hæmatin. On the other hand, chlorocruorohæmatin in ether-acetic has bands similar to those of hæmatin in the same solvent, but moved to the red.

15. Spectroscopically, chlorocruorochromogen closely resembles hæmochromogen. The former differs from the latter in that (1) its bands are nearer the red, (2) the final situation of the bands is not attained immediately after preparation; there is an initial form which gradually changes into the final one. The different specific chlorocruorins yield chlorocruorochromogens, all of which have the axis of the α -band at an identical wave-length.

16. Anson and Mirsky have demonstrated that the ammonia-hæmochromogens of hæmoglobin, heliocorubin, actinohæmatin and part of the cytochrome complex all have the axis of the α -band at the same wave-length, showing that the hæmatin group is either the same or very similar in all of these pigments. Ammonia-chlorocruorochromogen, on the other hand, has its α -band at a different wave-length, so that the hæmatin group of chlorocruorin is not the same as that of the pigments just mentioned.

17. The hæmatin group of the chlorocruorin molecule contains iron. It differs from the hæmatin group of hæmoglobin in that the iron is united to a different porphyrin. The latter has a similar spectrum to that of hæmatoporphyrin, with the bands shifted to the red.

REFERENCES.

- Anson, M. L., Barcroft, J., Mirsky, A. E., and Oinuma, S., 'Roy. Soc. Proc.,' B, vol. 97, p. 61 (1924).
Anson, M. L., and Mirsky, A. E., 'Journ. of Physiol.,' vol. 60, pp. 50, 161, and 221 (1925).
Barcroft, J., 'The Respiratory Function of the Blood,' Cambridge (1914).
Barcroft, J., and Barcroft, H., 'Roy. Soc. Proc.,' B, vol. 96, p. 28 (1924).

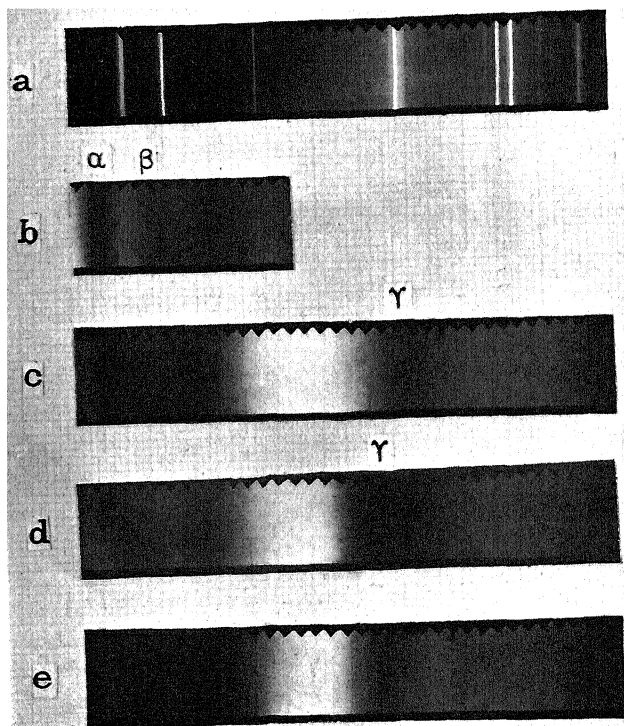


FIG. 2.

- Bertin-Sans, H., et Moitessier, J., 'C.R. Acad. Sci.,' vol. 116, p. 401 (1893).
 Dhéré, Ch., 'J. Physiol. et Path. gén.,' vol. 18, p. 221 (1919).
 Dhéré, Ch., et Sobolewski, S., 'C.R. Soc. Biol.,' vol. 70, p. 511 (1911).
 Fox, H. Munro, 'Proc. Camb. Phil. Soc. (Biol. Sci.),' vol. 1, p. 204 (1924).
 Hartridge, H., 'Journ. of Physiol.,' vol. 44, p. 1 (1912) and vol. 57, p. 47 (1922); 'Roy. Soc. Proc.,' B, vol. 86, p. 128 (1913).
 Henri, V., et Wurmser, R., 'C.R. Soc. Biol.,' vol. 72, p. 1039 (1912).
 Keilin, D., 'Roy. Soc. Proc.,' B, vol. 98, p. 312 (1925).
 Lankester, E. Ray, 'Journ. Anat. Physiol.,' vol. 2, p. 114 (1867), and vol. 3, p. 119 (1870).
 MacMunn, C. A., 'Quart. Journ. Micr. Sci.,' vol. 25, p. 469 (1885).
 Redfield, A. C., and Hurd, A. L., 'Proc. Nat. Acad. Sci.,' vol. 11, No. 2 (1925).
 Sorby, 'Quart. Journ. Micr. Sci.,' vol. 16, p. 76 (1876).
 Vlès, Fred, 'Arch. Phys. Biol.,' vol. 1, No. 1 (1921); vol. 2, No. 6 (1923); and vol. 4, No. 3 (1925).
 Winterstein, H., 'Bioch. Z.,' vol. 19, p. 384 (1909).
 Wurmser, R., 'Arch. Phys. Biol.,' vol. 1, No. 3 (1921).

DESCRIPTION OF PLATE 1.

FIG. 2.—Photographs of the absorption spectra of oxy- and reduced chlorocruorin of *Spirographis*. (a) Mercury vapour lamp spectrum. (b and c) Oxychlorocruorin. (d and e) Chlorocruorin. (b is photographed with a plate sensitive to the red.)

APPENDIX.

Spectrophotometric values from which the curves of Figs. 3 and 4 were constructed.

(Fig. 3—p. 204.)

Oxychlorocruorin.

λ in $\mu\mu$	Colog I/I ₀	λ in $\mu\mu$	Colog I/I ₀
638	0.282	537	1.061
616	1.896	517	1.086
609	2.45	502	1.064
580	0.994	491	1.259
561	1.450		

Reduced chlorocruorin

λ	Colog I/I ₀	λ	Colog I/I ₀
653	0.46(7)	573	1.434
630	0.76(9)	551	1.30
613	1.240	539	1.20
604	1.341	526	1.17
596	1.34(5)	514	1.10
590	1.380	509	1.02
577	1.367	496	0.96

(Fig. 4—p. 205.)

Oxychlorocruorin (oxygen).

λ	Colog I/I ₀	λ	Colog I/I ₀
460	0.50	350	0.56
450	0.66	340	0.64
435	0.74	322	0.68
430	0.74	310	0.70
420	0.66	295	0.86
410	0.60	285	1.14
400	0.54	278	1.30
395	0.44	275	1.35
390	0.46	268	1.18
385	0.48	261	1.27
380	0.51	250	1.45
375	0.48	245	1.57
368	0.48	242	1.66
360	0.43	238	2.24
358	0.42	234	3.74

Oxychlorocruorin (air).

λ	Colog I/I ₀	λ	Colog I/I ₀
450	0.54	310	0.39
440	0.69	300	0.42
430	0.67	290	0.55
420	0.42	285	0.70
400	0.30	278	0.81
395	0.18	274	0.77
390	0.23	267	0.75
380	0.27	261	0.79
370	0.31	254	0.85
360	0.28	245	0.97
350	0.29	240	1.38
340	0.31	234	1.80
321	0.36		

Reduced chlorocruorin.

λ	Colog I/I ₀	λ	Colog I/I ₀
465	0.23	425	0.41
450	0.68	420	0.29
440	0.69	410	0.30
430	0.44	405	0.23

[*Addendum, December, 1924.*—A further investigation has been made of the porphyrins derived from chlorocruorin.

Chlorocruoroporphyrin was first prepared by Nencki's procedure for the preparation of hæmatoporphyrin.* Diluted *Spirographis* blood was treated with acetic acid (1 part) plus ether (3 parts). The acetic was washed out with water and the remaining ether solution of chlorocruorohæmatin was evaporated to dryness. The residue (with some sodium sulphite, to prevent oxidation of the porphyrin) was dissolved in hydrobromic-acetic acid. The porphyrin thus formed was driven by sodium acetate into ether and then taken back into hydrochloric acid (1 part HCl plus 2 parts water). Hæmatoporphyrin, for comparison, was prepared from hæmin with hydrobromic-acetic acid. The axes of the bands of the two porphyrins (both in HCl, 1 in 3), measured with Hartridge's reversion spectrometer, were as follows :—

Nencki's hæmatoporphyrin	593	549
Chlorocruoroporphyrin (Nencki's method)	613	553

H. Fischer and O. Schumm ('Zeit. Physiol. Chemie,' numerous publications in recent years) have added much to our knowledge of porphyrins. They show that porphyrins fall into two groups.

Group I comprises porphyrins insoluble in chloroform. It contains : (1) Coproporphyrin, extracted from human fæces and from yeast by ether-acetic acid (Fischer and Schneller, 'Zeit. Physiol. Chemie,' vol. 130, p. 302 (1923), and Schumm, vol. 136, p. 243 (1924)); (2) Uroporphyrin, found in pathological human urine (Fischer, 'Zeit. Physiol. Chemie,' vol. 97, p. 125 (1916), and Schumm, *loc. cit.*); and (3) Turacin, the Cu salt of a porphyrin, found in the feathers of certain birds (Fischer and Hilger, 'Zeit. Physiol. Chemie,' vol. 128, p. 167 (1923)). Coproporphyrin is the porphyrin derived from a part of cytochrome (Keilin, *loc. cit.*). Cytochrome is present in relatively considerable amounts in yeast and in bacteria. Cytochrome (in yeast) gives, in addition, a porphyrin of Group II (Fischer, 'Zeit. Physiol. Chemie,' vol. 138, p. 288 (1924)).

Group II is formed by porphyrins soluble in chloroform. It contains (1) the porphyrin prepared from reduced hæmoglobin in whole blood by the action of HCl (Laidlaw, 'Journ. Physiol.,' vol. 31, p. 467 (1904), and Schumm, 'Zeit. Physiol. Chemie,' vol. 132, p. 34 (1924)); (2) Kämmerer's porphyrin, extracted from putrefying blood by ether and acetic acid (Fischer and Schneller, 'Zeit. Physiol. Chemie,' vol. 130, p. 302 (1923)); (3) Papendieck's porphyrin, obtain-

* I am indebted to Mr. R. Hill of the Biochemical Laboratory, Cambridge, for valuable help.

able from fæces after a meat meal (Papendieck, 'Zeit. Physiol. Chemie,' vol. 128, p. 109 (1923)); and (4) Oöporphyrin, extracted by HCl from egg-shells (Fischer and Kögl, 'Zeit. Physiol. Chemie,' vol. 131, p. 241 (1923)).

The following table shows the wave-lengths of the band-axes of the principal of these porphyrins :—

Porphyrin.	Reference.	Axes of bands in $\mu\mu$.					
		In ether.				In 25 per cent. HCl.	
GROUP I.							
Coproporphyrin	Fischer and Schneller, 'Zeit. Physiol. Chemie,' vol. 130	624	571	528	499	594	553
Ditto	Schumm, 'Zeit. Physiol. Chemie,' vol. 136	623	569	526	495	593	550
GROUP II.							
Laidlaw's porphyrin	Schumm, 'Zeit. Physiol. Chemie,' vol. 132	631	575	535	502	602	557
Kämmerer's porphyrin	Fischer and Schneller, 'Zeit. Physiol. Chemie,' vol. 130	633	575	533	498	602	557
Oöporphyrin	Fischer and Kögl, 'Zeit. Physiol. Chemie,' vol. 131	631	581	537	502	603	557

I next submitted chlorocruorin to a modification of Laidlaw's procedure for porphyrin preparation. *Spirographis* blood was treated with ether-acetic acid. The resulting chlorocruorohæmatin was taken into sodium carbonate solution and reduced with hydrosulphite. In a current of CO₂, conc. HCl was then added. The porphyrin thus formed was driven by sodium acetate into ether, and then taken back into HCl (1 in 3). For comparison, (1) the porphyrin from hæmin was prepared by a similar modification of Laidlaw's method, and (2) oöporphyrin was extracted from egg-shells with HCl. The measurements of the bands with Hartridge's spectrometer are given below :—

	Axes of bands in $\mu\mu$.	
	In ether.	In 1 part conc. HCl + 2 parts water.
Porphyrin from hæmin	632 585 536 503	600 555
Oöporphyrin	634 587 536 504	601 556
Porphyrin from chlorocruorohæmatin	641 580 553 512	614 559

It is seen that the bands of the porphyrin from chlorocruorin are situated considerably to the red of those of any of the above-mentioned porphyrins. This new porphyrin resembles the members of Group II in being soluble in chloroform.]

Effects of Ultra-Violet Radiation upon Involuntary Muscle, and the Supposed Physiological Interference of Visible Rays.

By Y. AZUMA and LEONARD HILL, F.R.S.

(Received November 11, 1925.)

(From the National Institute for Medical Research.)

It was shown by Adler (1) that involuntary muscle is excited and its tone increased by radiation with ultra-violet rays. The excised frog's stomach, the bladder and the uterus of the rabbit, and that of the guinea-pig, were suspended by him, each in a suitable salt solution, and excited by the mercury vapour lamp. With a glass screen interposed no result was obtained, the visible and longer ultra-violet rays, *e.g.*, those longer than about 3200 A.U., having no exciting effect.

There may be recalled the old observation of Lambert (1760) that the excised iris of fish and frogs reacts to light by contraction; the visible rays in this case act through the pigment which absorbs them (Steinach, Hertel). Contraction of the pigment-free ventral band of the earth-worm can be produced by ultra-violet, but not by visible rays. On the other hand, visible rays excite the pigmented ventral band of *Sipunculus nucleus* (Hertel, 2). Probably the nerve plexus is excited by the conversion of visible rays into heat in the pigment cells which absorb these rays.

According to Adler, ultra-violet rays must act directly on involuntary muscle, because they equally excite organs which are normally inhibited by excitation of the sympathetic nerve supply and those which are excited by this means; moreover, emetin arrests the excitation of involuntary muscle by ultra-violet radiation, and this is believed to act on the muscle (Pick and Wasicky, 3). Involuntary muscle sensitised by hæmatoporphyrin, or eosin, is thrown into a state of increased tone by visible rays, these rays being absorbed by tissue which has combined with the sensitising substances.

It is of interest to note that O. Bernhard observed in man increased peristalsis of the bowel, in cases of laparotomy directly exposed to the sun, and even with intact abdominal wall when this was thin, or in which there was an abdominal hernia.

A. Eidinow and L. Hill have observed the active peristalsis of the exposed bowels in rabbits put under the mercury vapour lamp, and suggest that such radiation might possibly be useful in preventing paralysis of the bowel movement, which so often results from operation on the abdomen. The bowels might be radiated sufficiently to start active peristalsis before closure of the wound.

D. T. Harris (4) has claimed recently that while the ultra-violet rays, using blue uviol glass as a screen, excite, these rays *plus* the visible rays coming from the naked mercury vapour lamp do not excite the frog's stomach, and in consequence puts forward the view that there is a physiological interference between the visible and ultra-violet rays. A. Eidinow and L. Hill thought they had obtained some evidence that infusoria moved actively a little longer when radiated with ultra-violet *plus* visible rays than with the latter alone, but further research showed them that immobility took place after the same duration of exposure in both cases, and that there was no evidence of physiological interference, a conclusion which was confirmed by observations of Argyle Campbell and L. Hill (5) on the circulation in the exposed mesentery of frogs and mice. Hess (6) has put forward evidence in favour of physiological interference derived from the use of glass screens, one of which transmitted ultra-violet rays, and the other these rays (claimed to be the same) together with visible rays. Longer exposure each day to the mercury vapour lamp was required to protect rats, fed on a deficient diet against rickets, when the second screen was used, than was necessary in the case of the first screen. It is, however, impossible merely by spectrograph photographs to determine whether the ultra-violet intensity was the same in the two cases, and the most probable explanation of Hess's results was inequality of ultra-violet intensity.

In the present research, the following organs were investigated: frog's stomach and large intestine, rabbit's large intestine, guinea-pig's non-pregnant uterus and large intestine.

The apparatus used consisted of a small chamber bounded by a quartz plate on one side and a glass plate on the other side, each 3 mm. thick. This chamber was filled with 20-30 c.c. of a suitable nutritive fluid, and in this the preparation was suspended, attached below to a glass hook and above to one arm of a light lever, the other arm of which was fitted with a suitable style which wrote upon a smoked drum. Oxygen was bubbled through the nutritive solution, and in the case of mammalian organs this was kept at body temperature by immersing the lower half of the chamber in a bath of warm water.

For the frog's large intestine we used Tyrode's solution.

(1) NaCl	6.5	(2) NaH ₂ PO ₄	0.05
K Cl	0.2	NaHCO ₃	1.0
CaCl ₂	0.2	H ₂ O	200.0
MgCl ₂	0.1		
H ₂ O	800		

(1) and (2) being mixed shortly before use.

For the mammalian organs we used a solution with the following composition :—

NaCl	9
KCl	0.42
CaCl ₂	0.24
MgCl ₂	0.005
NaHCO ₃	0.5
dextrose	0.5
H ₂ O	1000

For the frog's stomach this solution was diluted so as to give a concentration of 0.6 per cent. NaCl. We find that the presence of calcium is necessary for the increase of tone produced by ultra-violet rays.

As source of ultra-violet rays we used a Kelvin, Bottomley and Baird water-cooled mercury vapour lamp, with and without a blue uviol glass screen. The lamp takes 2.5 ampere and 100–110 volts across the electrodes. As the source of visible rays, we used a small 5-ampere carbon arc, fitted with a focussing lens, and cut out those ultra-violet rays which are biologically active by passing the light through the glass side of the chamber, interposing a water screen to remove the dark heat rays.

The visible rays had no effect on the tone or rhythmical contraction of any of the organs investigated, under normal conditions, but excited increased tone when the organs (*e.g.*, large intestine of frog, uterus of guinea-pig) were sensitised with hæmatoporphyrin 1 : 100,000 to 1 : 50,000. The tone then increases to a maximal degree and persists, all rhythmical contraction ceasing. The hæmatoporphyrin had no effect so long as the organs were shaded from light. Eosin 1 in 10,000 had poisonous effect on the large intestine of the frog, and this prevented the above sensitising effect on the tone being manifested.

The effect of ultra-violet rays.—Blue uviol glass lets through the blue end of the visible spectrum, the longer ultra-violet rays, and some of the biologically active ultra-violet rays, viz., rays shorter than 3100 ; but it both cuts out the shorter rays and cuts down the intensity of the middle ultra-violet rays very greatly. Thus, the mercury vapour lamp screened by blue uviol glass takes about ten times as long to kill infusoria in a quartz cell, as when not so screened. The frog's stomach, used by D. T. Harris in his observations, we found to give very irregular spontaneous contractions and alterations of tone, and, therefore, to be far less suitable for an investigation on the supposed physiological interference of visible with ultra-violet rays than the other organs studied by us. In most cases irradiation through blue uviol glass produced no effect on the stomach, while that with the unscreened mercury vapour lamp excited increased tonus.

No evidence of a physiological interference between visible and ultra-violet rays was found by us, but many experiments failed to be convincing owing to the lack of response or to the irregular spontaneous contractions and relaxations of this organ. The frogs we used were summer and autumn ones. The large intestine of the frog, on the other hand, is an excellent organ, most reliable in its behaviour, and offering a thin surface for the action of ultra-violet rays in contrast to the thick-walled stomach.

With the blue uviol glass screen the ultra-violet rays slightly increase the tonus of this organ, and the frequency of the natural rhythmical contractions, while the unscreened mercury vapour lamp strongly increases the tonus, and these effects are not at all inhibited by concomitant illumination of the intestine with visible rays from the carbon arc.

The guinea-pig's non-pregnant uterus sometimes does not respond to radiation through blue uviol glass, and sometimes shows increased tonus, while with the unscreened mercury vapour lamp a largely increased tonus results, with at first greater frequency of rhythmical movements, but ending in a state of tonic contraction. Here, again, visible rays from the carbon arc had no inhibitory effect upon the exciting effect of the rays from the mercury vapour lamp. So, too, with the large intestine of the rabbit and guinea-pig. Like results were obtained with fourteen preparations of uterus and eighteen of large intestine.

Our conclusion then is directly negative to that of D. T. Harris. No physiological interference occurs between visible and ultra-violet rays. Further, if visible and dark heat rays together are concomitantly thrown with the ultra-violet rays, upon the large intestine or uterus, which are kept cool by irrigation, we find no interference occurs.

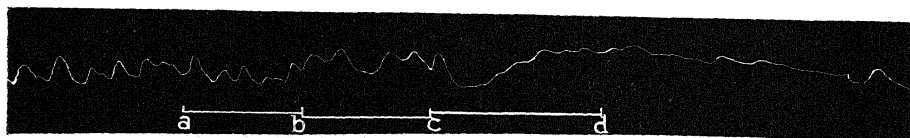


FIG. 1.—Frog's stomach. *a-b*, M.V.L. through ordinary glass. *b-c*, M.V.L. through blue uviol glass. *c-d*, naked M.V.L.

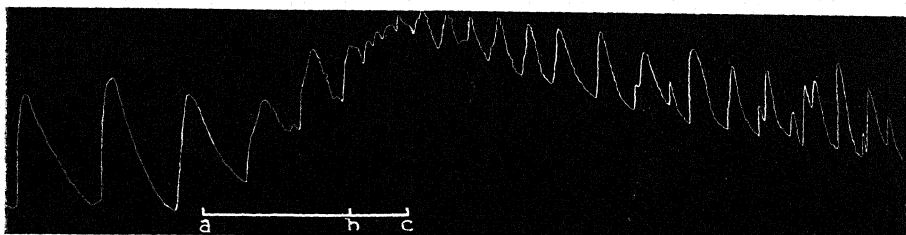


FIG. 2.—Frog's rectum. *a-b*, M.V.L. through blue uviol glass ; *b-c*, naked M.V.L.

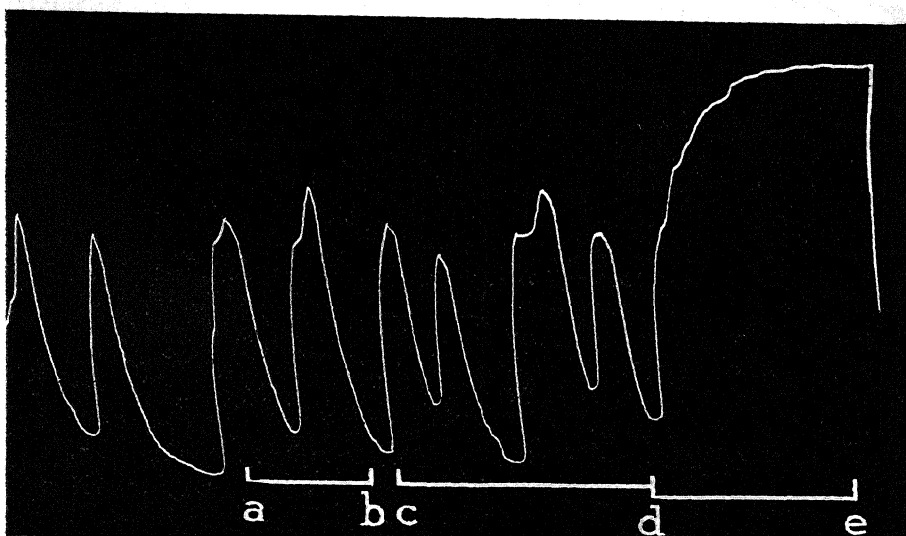


FIG. 3.—Guinea-pig's uterus. *a-b*, C. Arc through ordinary glass ; *c-d*, M.V.L. through blue uviol glass ; *d-e*, naked M.V.L. ; *e*, drum stopped—relaxation followed slowly.

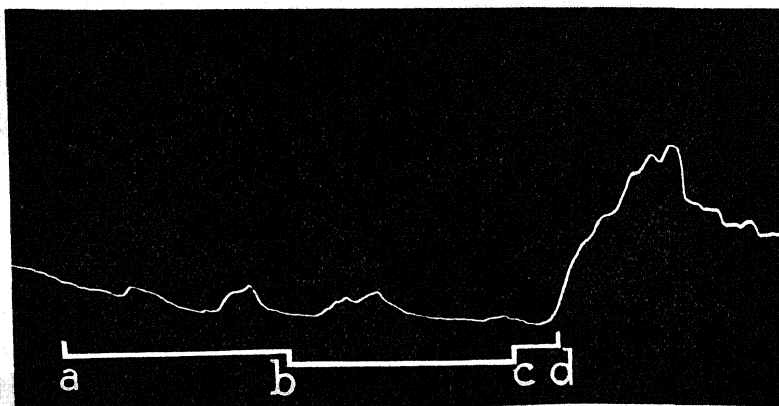


FIG. 4.—Guinea-pig's uterus. *a-b*, M.V.L. through blue uviol glass + C. Arc through ordinary glass ; *b*, C. Arc off, M.V.L. through blue uviol glass ; *c-d*, naked M.V.L.

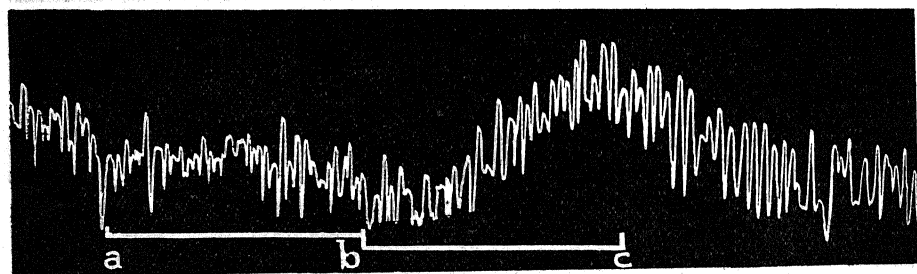


FIG. 5.—Rabbit's large intestine. *a-b*, C. Arc through ordinary glass ; *b-c*, naked M.V.L.

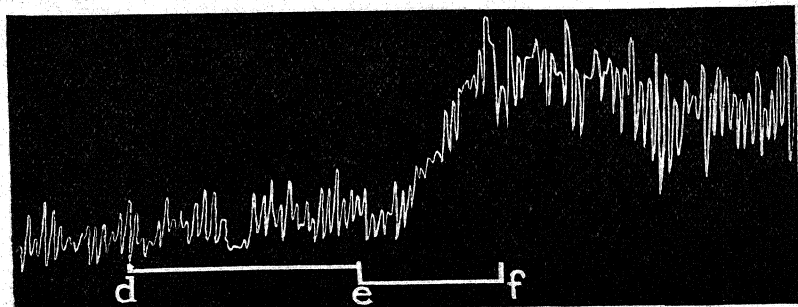


FIG. 6.—Rabbit's large intestine. *d-e*, M.V.L. through blue uviol glass ; *e-f*, naked M.V.L.

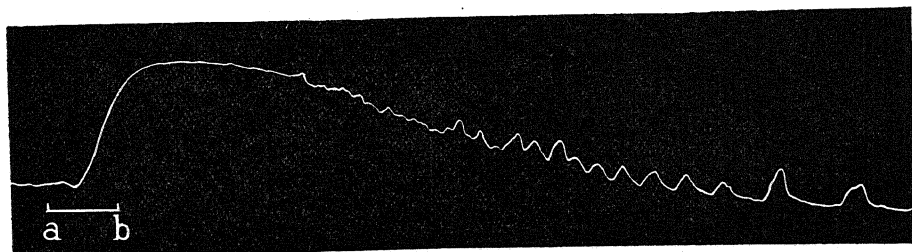


FIG. 7.—Guinea-pig's uterus sensitized by 1 : 100,000 h matoporphyrin solution. *a*–*b*, C. Are through ordinary glass.

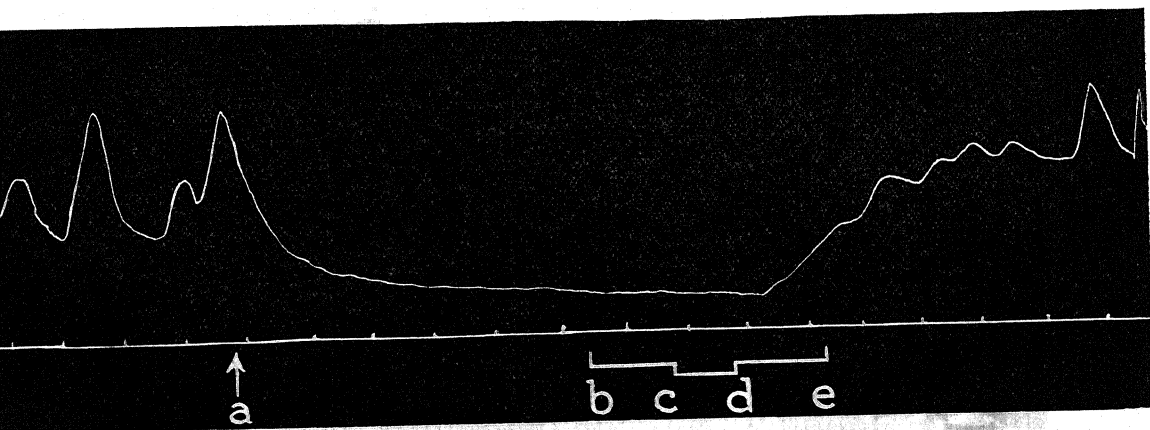


FIG. 8.—Frog's rectum. *a*, 1 : 2,000,000 adrenalin hydrochloride solution ; *b*–*c*, M.V.L. through ordinary glass ; *c*–*d*, M.V.L. through blue uviol glass ; *d*–*e*, naked M.V.L. Time in minutes.

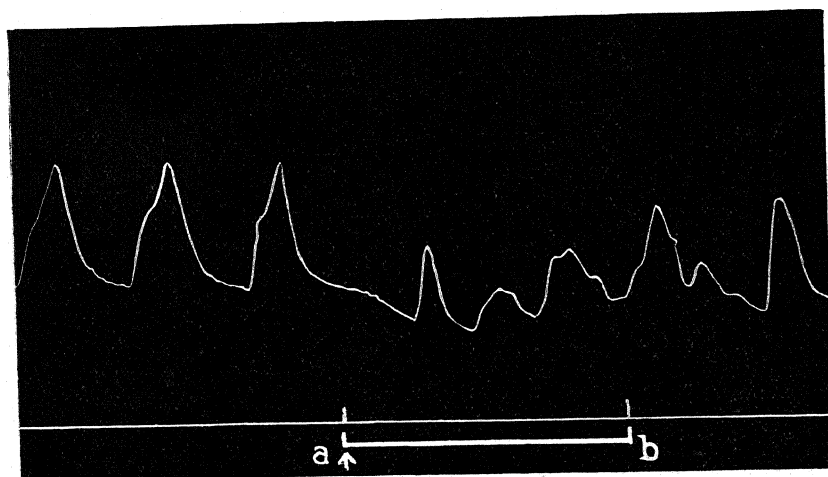


FIG. 9.—Frog's rectum. *a*, 1 : 2,000,000 adrenalin solution and simultaneous irradiation with the naked M.V.L. (*a*–*b*).

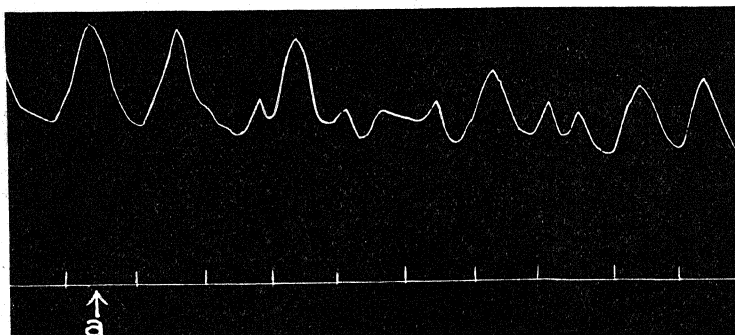


FIG. 10.—Frog's rectum. *a*, 1 : 40,000,000 adrenalin solution which had been irradiated with the M.V.L. in a quartz flask for two hours. Time in minutes.

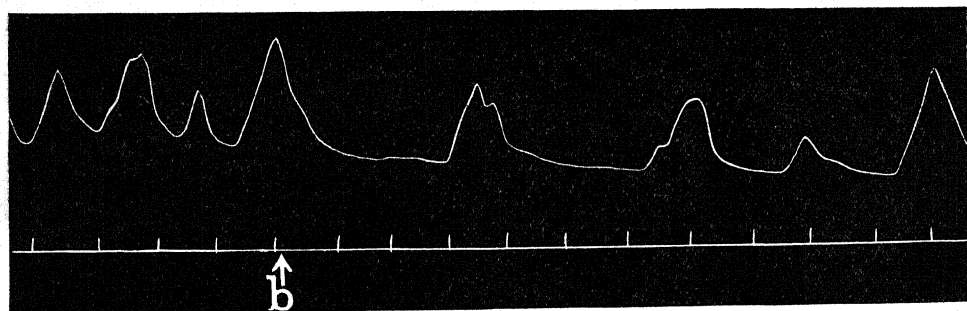


FIG. 11.—*b*, 1 : 40,000,000 adrenalin solution which had been irradiated in an ordinary glass flask. Time in minutes.

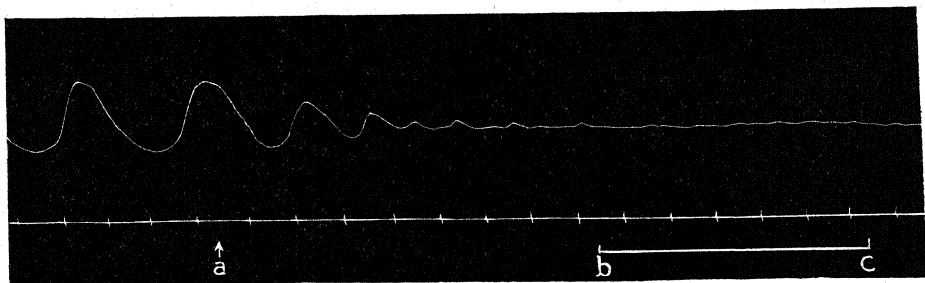


FIG. 12.—*a*, 1 : 2,000 emetin hydrochloride ; *b-c*, naked M.V.L. Time in minutes.

We have found that, while the large intestine of the frog is relaxed by adrenalin hydrochloride 1:2,000,000, this relaxation is antagonised by irradiation with the mercury vapour lamp. If the intestine be in a state of tonic contraction excited by the lamp, adrenalin has no relaxing effect; relaxation is slight if the intestine be exposed to adrenalin and the lamp at one and the same time; the intestine relaxed by adrenalin recovers tone rapidly when radiated.

By rotating adrenalin (1 in 40,000,000) in a quartz flask (water-cooled) in front of the mercury vapour lamp, it becomes oxidised and reddish in colour, and loses a large part of its relaxing power.

Emetin 1 in 2000 stops the rhythmic contractions of the large intestine—an irreversible effect and radiation does not then excite. Emetin solution fluoresces strongly, but does not absorb the biologically active rays.

Figs. 1-12 illustrate the above results.

Summary.

No physiological interference is produced by dark heat or invisible rays with the exciting action of ultra-violet on involuntary muscle. The action of ultra-violet rays antagonises that of adrenalin on involuntary muscle, but not that of emetin. The presence of calcium in the nutritive fluid is necessary for the increase of tone produced by ultra-violet rays.

We are indebted to Dr. H. H. Dale for advice in the method of recording the contractions of the guinea pig's uterus.

REFERENCES.

- (1) Adler, 'Archiv f. exper. Path. u. Pharmacol.,' vol. 85, p. 152 (1920).
 - (2) Hertel, 'Ztschr. f. allg. Physiol.,' vol. 6, p. 44 (1904).
 - (3) Pick and Wasicky, 'Archiv f. exper. Path. u. Pharmacol.,' vol. 80, p. 147 (1917).
 - (4) Harris, 'Proc. Roy. Soc.,' B, vol. 98, p. 171 (1925).
 - (5) Campbell and Hill, 'Brit. J. Exper. Path.,' vol. 5, p. 317 (1924).
 - (6) Hess, Pappenheimer and Weinstock, 'Proc. Soc. Exper. Biol. and Med.,' vol. 20, p. 14 (1922).
-

The Early Development of Cavia: Note on Associated Remains of Previous Placentation.

By NORMAN H. W. MACLAREN.

(Communicated by Prof. T. H. Bryce, F.R.S.—Received November 18, 1925.)

(From the Embryological Laboratory, Anatomy Department, University of Glasgow.)

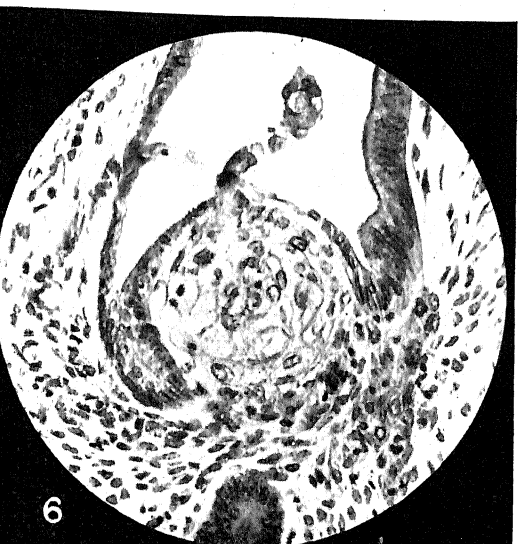
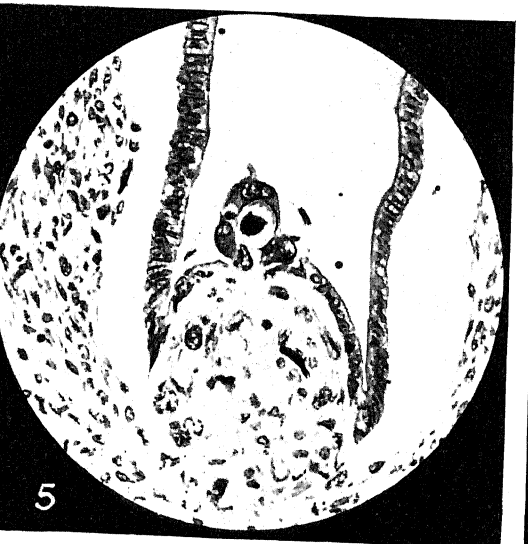
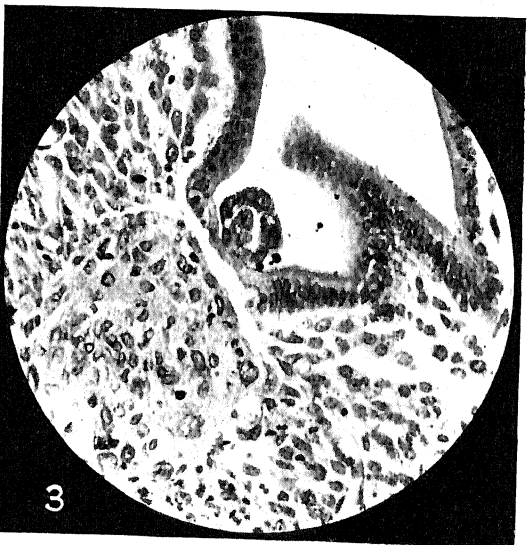
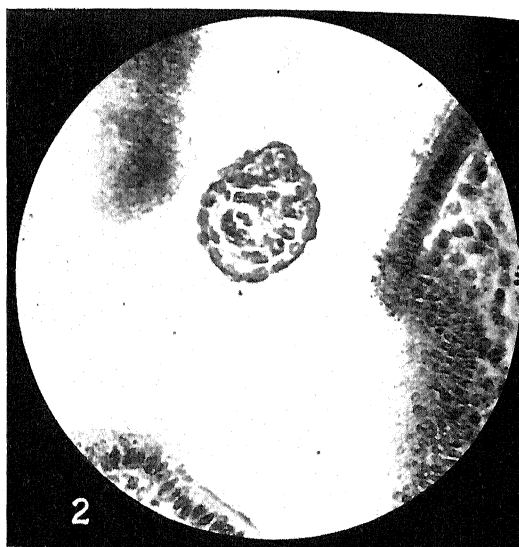
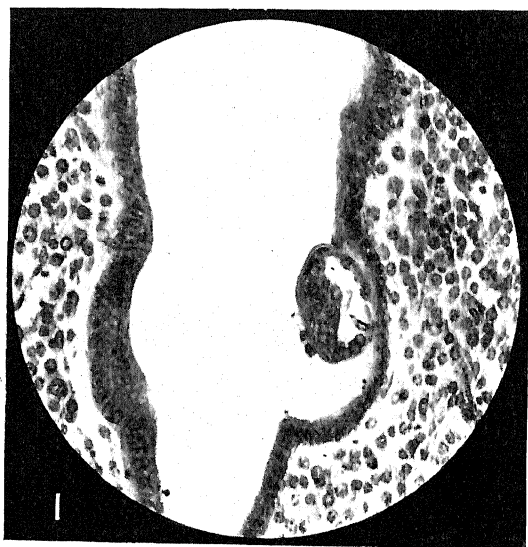
[PLATE 2.]

While investigating the early development of *Cavia*, some phenomena were observed which, when considered with reference to the implantation of the ovum, seem to be of very considerable significance. It is not proposed in this preliminary note to deal with the actual implantation process in *Cavia*, but simply to describe and illustrate these remarkable appearances and briefly to explain their origin.

Fig. 1 (Plate 2) is a young blastocyst with the zona intact, lying, as free blastocysts usually do, in a slight and partial pouch of the ante-mesometric wall of the uterine cavity. It is noticeable that there is no reaction in the maternal tissues. Fig. 2 is a slightly older blastocyst with the zona now gone, and the central mass differentiated from the trophoblastic mass or *träger*. Here, also, there is no reaction in the maternal tissue. These two embryos may be regarded as normal, and are two out of a considerable series in my possession. They definitely demonstrate the fact that implantation does not occur at a stage that is any younger than that represented by the elder of these two.

The appearances to which it is desired to draw special attention are represented in figs. 3, 4, 5 and 6. These belong to a numerous group of structures, which vary a good deal, both in size and in character, but can all be shown to be placental fragments, which have lingered from the previous pregnancy, and are either in process of being thrown off by the regenerating epithelium or included by it, as tiny bodies which will eventually degenerate and disappear.

The similarity that some of these bear to actual embryos is at first perplexing. The section shown in fig. 4, for example, when examined alone out of its series is very deceptive. A group of cells lies next the thinned-out epithelium, while the sub-epithelial maternal tissue shows what looks like a distinct "reaction." When, however, the whole series is examined, the group of cells proves to be nothing more than a small placental fragment in



process of extrusion, while the reaction area is merely a piece of an old placental site.

As is to be expected, these placental remnants are most frequent on the mesometric side of the uterus, *i.e.*, the side to which the placenta was attached. Figs. 3, 5 and 6 are from that position. But quite frequently one finds them lower down in the ante-mesometrium (fig. 4), a fact no doubt to be accounted for by the relatively enormous size of the full-grown placenta.

Bodies of this nature do not seem to have been previously recognised in *Cavia*, and their possible presence in the same uterus as normal embryos necessitates careful discrimination. As was to be expected, I have not found any trace of such structures in the virgin uterus. Traces of them are still to be found at the 76th day after parturition.

DESCRIPTION OF PLATE 2.

FIG. 1.—A blastocyst lying free in a pocket of the uterine cavity. The zona radiata is intact, three cells from the corona radiata are still adherent to it. The trophoblast consists of a peripheral layer with a thickening at one pole. ($\times 200$.)

FIG. 2.—A slightly older now bipolar blastocyst free in the uterine cavity. The zona radiata has disappeared, only traces remaining. The central cell mass is now distinct. The trophoblast is thickened at one pole of the blastocyst (the future *träger*). ($\times 200$.)

FIG. 3.—Knob of cells resembling an embryo lying in a pocket of the epithelium in the mesometric section of the uterine cavity. The sub-epithelial tissue shows a mass of cells sharply differentiated from the remainder of the decidua. In a neighbouring section the knob and the sub-epithelial mass are continuous as in fig. 5. The mass includes cells with massive bodies and large nuclei and also cells in various stages of degeneration. These elements exactly resemble those met with in a section of a shed placenta. ($\times 200$.)

FIG. 4.—Similar to fig. 3, but from the lateral wall of the uterus. The epithelium forms a lamella beneath the knob of cells. In the sub-epithelial mass are seen nuclei of the large cells mentioned under fig. 3; also a mitotic figure which suggests reaction. The knob of cells occurs in 8 sections ($10\ \mu$ thick). The sub-epithelial mass retains the form seen in this figure in 22 sections. ($\times 200$.)

FIGS. 5 AND 6.—Sections through the mesometrical end of the uterine cavity. The sub-epithelial masses have the same characteristics as described under figs. 3 and 4. But in both the uterine epithelium is regenerated over the sub-epithelial mass and appears to be cutting off a knob of cells corresponding to those seen in figs. 3 and 4. ($\times 200$.)

Studies on the Relation of Gonadic Structure to Plumage Characterisation in the Domestic Fowl.—I. Henny-Feathering in an Ovariectomised Hen with Active Testis Grafts.

By A. W. GREENWOOD and F. A. E. CREW, Animal Breeding Research
Department, University of Edinburgh.

(Communicated by Prof. R. C. Punnett, F.R.S.—Received Nov. 24, 1925.)

[PLATES 3 AND 4.]

CONTENTS.

1. Introduction	232
2. Description of Specimen	233
3. Histological Findings	234
4. Discussion	236
5. Summary	240
6. References	240
7. Description of Plates	241

Introduction.

In certain breeds and varieties of the domestic fowl, *e.g.*, the Brown Leghorn, the adult plumage presents a sexual dimorphism in the kind and distribution of colour in the individual feathers, and in one or more of the seven areas in which plumage colour differences may distinguish breed from breed, and variety from variety, and also in the structure of the feathers of the neck and saddle hackles, and by the presence of the large tail sickles of the male. In other cases, such as the White Leghorn, the sexes are to be distinguished only by the structural differences in the hackle feathers and by the large tail sickles of the male. In the case of certain other breeds, the Campines and the Sebrights, for example, the plumage of the male is identical with that of the female both in colouration and in structure.

Cocky-feathering in the case of such varieties as the Brown and the White Leghorns can be regarded as a trustworthy indication that within the body there is, or was at the time when the plumage was developed, active functional testicular tissue; henny-feathering as an indication that there is, or was when the plumage was developed, active functional ovarian tissue. Gonadectomy in both sexes is followed after a moult by the assumption of a plumage which has the colouration of that of the male of the variety to which the bird belongs, whilst the barbules in the distal portions of the feathers of the hackle regions are absent, also a characteristic of the cocky-feathered male, but the plumage

is much looser and far more luxuriant in its growth; the plumage characters of the capon and of the poularde are exactly alike. Since this is the case, it is commonly argued that the gonads exhibit an endocrine function, the ovarian hormone possessing the faculty of inhibiting the development of cocky-feathering. The fact that in the Campine and the Sebright the cock is also henny-feathered is explained on the assumption that in their functioning the testes of such a male are equivalent endocrinologically to the ovary of a hen. Such an interpretation is supported by the facts that castration of the normally henny-feathered cock is followed by the assumption of typical cocky-feathering; that castration of the normally cocky-feathered male and subsequent successful ovarian implantation is followed by the development of henny-feathering; and that, in such cases as have so far been recorded, successful testicular implantation in an ovariectomised hen has been followed by the development of a plumage of the cock (as opposed to that of the capon or poularde). There is undoubtedly a considerable body of evidence, secured from careful experimentation, that supports the endocrine interpretation of the relations between gonad structure and plumage characterisation, and most biologists have accepted this interpretation. However, there are several facts concerning the fowl that cannot easily be reconciled to this hypothesis. The case about to be discussed in our opinion, severely questions its validity.

Description of the Specimen.

The bird, one of the series used by Finlay (1925), was left by him with us because of its patent interest and importance, in order that a complete examination of it could be made. We wish to express our thanks to our late colleague for the pleasure that has been ours during the course of this study.

The ovary of a Brown Leghorn female was removed when the chick was four days old, and immediately following this the chopped-up testes from a brother (i.e., a Brown Leghorn, and destined to become cocky-feathered) of the same age was inserted on the left side of the body cavity just anterior to the kidney. The operation was entirely successful, and the recovery uneventful. When the adult plumage was assumed, it was as that of the normal Brown Leghorn cock. The bird was a perfect specimen of the masculinised female, with large, erect comb, large earlobes, large pendulous wattles, and developing spurs, distinguishable from the real male only by the smaller size of its skeleton (Plate 3, fig. 1). Though it behaved as a male in its relations with normal hens it was sterile. At the age of seventeen months, following a complete moult, its plumage became as that of a Brown

Leghorn hen (fig. 2). In every other respect it remained as it had been before the moult. It was kept under observation for a further seven months, and during this time any feather replacing an older one was definitely and entirely henny.

At the time of the post-mortem examination, the comb, as large and as turgescient as that of a normal male of this variety, measured 10×7 cm.; the wattles 6.5×5 cm. The spurs were 1.75 cm. long. The bird was entirely healthy and in excellent condition.

Four masses of gonadic tissue were present—two on either side. On the left there was a large trilobed mass (fig. 3), two lobes of which were manifestly testicular, whilst the third when incised proved to be a sac distended with fluid resembling the male ejaculate. This mass was regarded as part of the testis tissue implanted two years previously. Ventral to this graft, which had so abundantly multiplied itself, was a smaller mass in the normal position of the gonad. This was regarded as an ovarian fragment left behind at the time of operation and thereafter enlarged. On the right side of the body a small bean-shaped mass of testicular tissue was attached to the mesentery. This was regarded as another survival of the testicular implantation. There was also a smaller mass of what appeared to be testicular tissue, measuring 0.75×0.3 cm., in the gonad site close to the right adrenal, and regarded as a resuscitated right gonad. The four masses of gonadic tissue were removed for sectioning, after it had been demonstrated that on neither side did there exist any appreciable connection between the testicular tissue and the vasa deferentia, of which the one on the left was well developed with prominent convolutions in its caudal position, whereas that on the right side was much thinner and more delicate with no convoluted portion. It is noteworthy that following the implantation of testicular tissue and the differentiation of the right gonad into a testis the vasa deferentia became further developed, and also that the larger vas was on the side on which the greater mass of testicular tissues lay. No trace of an oviduct was found on the right, but on the left there was a small one, which had retained its infantile proportions.

Histological Findings.

The gonadic tissue was fixed in Allen's Bouin at 38° C. Sections were prepared and stained by Heidenhain's iron-alum hæmatoxylin method.

The posterior lobule of the large testis graft (fig. 3, A) was composed of a thin capsule filled with a milky fluid, which on microscopical examination was found to contain large numbers of motile spermatozoa. Sections of the

median and anterior lobules (fig. 3, B and C) revealed a typical testicular structure (figs. 4 and 5) consisting mainly of large swollen tubules packed closely together, in which all stages of spermatogenic activity were represented. Large numbers of mature spermatozoa were found in the central area of the tubules. The intertubular spaces were very restricted, and contained only a few cell elements, as is the case in the functioning testis of the normal cock. Among the tubules in which spermatogenesis was actively proceeding there were other tubules of varying sizes, with a somewhat thickened basement membrane which had a scanty lining of columnar epithelial cells. In many of these tubules the cells of the germinal epithelium had been desquamated. The cavity of the tubules was filled with fluid containing numerous mature sperm and much cell debris. Earlier stages in the degeneration of the spermatogenic tubules were represented by a few tubules in which the epithelial lining was regular. The component cell nuclei were all in the resting stage. The succeeding cell layers towards the centre of the tubules were regular, but showed various stages in nuclear degeneration. Examination of the tissue ventral to this large testicular mass (fig. 5, D) revealed a typical ovarian structure (fig. 6). The ovariectomy had been incomplete. The ovarian tissue was definitely degenerate. On the surface of the mass were numerous clusters of cystic follicles of varying size containing a thin fluid, whilst embedded in its stroma there were one or two small oocytes of normal appearance (fig. 7). The stroma of the ovary was invaded by numerous cellular cords, between which strands of a deeply staining substance were found, which probably represented the final stage in the degeneration of some of the cords. There had been a heavy infiltration of masses of granulocytes in the peripheral regions of the mass. No evidence of the differentiation of the cords into tubules was encountered.

The testis graft from the right side was found to possess a structure similar to that of the testicular tissue on the left. There were large swollen tubules in active spermatogenesis in which many mature sperm were present, and other somewhat smaller tubules with a distinct central lumen, and with a lining layer of germinal epithelium in which the meiotic phases were completely absent. Tubules packed with mature sperm and cell debris were also encountered.

The tissue occupying the right gonad was divided up into four distinct areas by bands of fibrous tissue. There was a relatively large area composed wholly of tubules in active spermatogenesis; all meiotic phases were represented and numerous mature sperm were found in the central

regions of the tubules, which were surrounded by a thick layer of fibrous tissue.

A somewhat smaller area was composed mainly of young epithelial cords embedded in a connective tissue stroma. Scattered among the cords were a few small tubules with a well-marked central lumen, and with a layer of columnar germinal epithelium lining the basement membrane. These resembled in structure and general appearance the testicular tubules normally found in the immature cock.

A third area was composed of separate lobules, which consisted either of a collection of young sex cords, enlarged sex cords and atrophic tubules (tubules with a scanty lining of epithelial cells with fibrillar protoplasm stretching across the central lumen), or entirely of atrophic tubules (fig. 8).

At the periphery of this area was another consisting of large tubules characterised by the presence of a wide irregular lumen and a rather shallow columnar epithelial lining which resembled parovarian rather than spermatid tubules. Centrally, the area was composed largely of enlarged sex cords and tubules of an immature type similar to those already described. In this region the tubules were separated one from another by invading strands of fibrous tissue (fig. 9).

Discussion.

Here, then, is an individual born a female and subsequently masculinised by ovariectomy and testis implantation, in which there was a fragment of highly degenerate ovarian tissue and a mass of testicular tissue much larger in relation to the weight of the body than are the testes of the normal cock, and which, having been cocky-feathered previously, had later assumed the plumage characterisation typical of the normal hen of her breed and variety.

It is reasonable to hold that the history of the gonadic tissue was as follows:—

(1) The ovarian tissue on the left side of the body was almost, but not quite, completely removed at the time of the operation. The portion left behind had continued to develop and had increased in size only to undergo degenerative changes later. There was nothing in this tissue that could support any suggestions that there had been regeneration of ovarian tissue after complete ovariectomy. The fact that a few oocytes of normal appearance were found can readily be explained on the assumption that there is a time seriation in the ripening of the follicles, and that follicular atresia occurs only when the follicle attains a certain minimum degree of development. The normal oocytes found are to be regarded as the last of those included in the ovarian fragment left behind at the time of operation.

(2) The right gonad of this bird had ceased its development and differentiation on about the eighth day of incubation, as is the case in all female chicks. Following the removal of the left gonad, the differentiated ovary, this right gonad had continued its development. Its differentiation, however, occurred under conditions of the internal environment quite distinct from those under which the left gonad had become ovarian in structure. These conditions were such as to encourage the differentiation of the developing yet still ambivalent right gonad into testicular tissue or/and as to discourage its differentiation into ovarian. Thus, though by virtue of its genetic constitution the component tissues of this right gonad were preferentially ovarian, they, being not yet completely differentiated, under the directing stimulus of the internal environment, which may be defined as the peculiar state established by the action and interaction of the physiological processes of the body as a whole, became typically and functionally testicular. The fact that the previously atrophic right gonad, following ovariectomy in the hen, becomes a testis has been frequently recorded (Benoit, 1923 ; Domm, 1924 ; Pézard, 1918 ; Zavadovsky, 1922).

(3) The testis grafts had secured attachment and had flourished, undergoing a very considerable enlargement. To record the actual weight of the testicular tissue implanted and found post-mortem is not of interest, in view of the fact that the size of the gonad of the fowl varies so very markedly with changing conditions. Mild sickness, for example, is attended by a marked decrease in the size of the gonad, and the change in size occurs with almost startling suddenness. It is enough to state that the mass of testicular tissue, in relation to the weight of the body, was greater than is that of the testes of the average normal cock.

At the time of the assumption of the adult plumage this hen became completely cocky-feathered. If no gonadic tissue had been present during the critical period in the development of this plumage, this would have been as that of a poularde, cocky in colouration and structure, but as that of the agonadic bird in the length of the feathers, its looseness, and luxuriance. If at the beginning of this critical period no, or insufficient, gonadic tissue had been present, then the plumage would have started to develop the characterisation of the plumage of the poularde, but if shortly after this gonadic tissue of either kind, ovarian or testicular, had become sufficiently active, then though in its colouration and structure the plumage would remain unaffected it would have become tighter and closer, to simulate exactly that of the cock.

It follows from this that at the critical time of the development of the first adult plumage ovarian tissue in the body was either absent or insufficient, and that almost immediately following this either ovarian or testicular tissues became sufficiently active physiologically, or else that ovarian tissue was either absent or insufficient throughout, but that sufficient testicular tissue was present either at the beginning or else immediately after. If the bird was a poularde, the plumage of which in the later stages of its development had become affected by the physiological activity of functional ovarian tissue, then after a moult the plumage would have become completely henny, as indeed it did, the head furnishings would have always been as those of a female of this breed and variety, which they were not, and only ovarian tissue would have been found at the time of death, which was not the case. Both ovarian and testicular tissues were found post-mortem, and it is, therefore, reasonable to hold that during the critical period in the development of the first adult plumage the ovarian fragment was insufficient to affect its differentiation, and that before the implanted testicular tissue could exert any influence it required a certain time to secure vascular connections and to increase in size. The first adult plumage began its differentiation in an internal environment equivalent to that of a poularde, but the testicular grafts, having secured a hold and flourished, came into play in time to tighten the plumage and limit its growth. The plumage became cocky, the head furnishings as those of the typical male.

Since during the first year of its life the sporadic moulting of individual feathers was followed by the development of others typically male in colour, there was never any reason to assume that there was any ovarian tissue at all or any considerable amount of this in the bird. Yet following the annual moult the entire plumage became completely henny, though the head furnishings remained as those of the male. In the case of such a variety as the Brown Leghorn the only gonadic condition which is associated with henny plumage and male type head furnishings and behaviour is that of ovarian tumour in the hen. Post-mortem examination revealed no such tumour but a very large amount of functional testicular tissue. There is no reason whatsoever for suggesting that the condition of the gonadic tissues at the time of the development of this plumage was essentially different from that found at the time of death. There perhaps may have been rather more than less testicular material and slightly more ovarian, but essentially the condition of the gonadic tissues was as that found post-mortem.

If this is indeed the case, it is necessary to explain the association of abundant

testicular tissues and henly-feathering. It was not testicular material from a normally henly-feathered cock that had been implanted, so that this argument is not available. It would seem that the only explanation that can fit the facts is that which postulates that ovarian and testicular tissues, in respect of their own individuation, exert demands upon the general economy of the same kind, but different in degree; that the functioning of an ovary is physiologically more expensive than is that of the testes (Smith, 1910), and that it is possible to supplement the demands of the testes so that they become equivalent to those of the ovary. Such a metabolic hypothesis is in general agreement with the conclusions of Riddle (1925) concerning the phenomena of sexual differentiation, and is in line with the much older one of Thomson and Geddes (1889). It has been shown by Finlay (1925) that if in the body of either a cock or of a hen both functional ovarian and testicular tissues are present in any considerable amount the plumage is henly (though it is to be noted that, in the case of the Brown Leghorn, this henly plumage is distinctly more highly coloured, particularly in its red, than is that of the normal hen of this variety). This can readily be explained on the hypothesis suggested; the augmentation of ovarian tissue produces no effect, since the plumage cannot become more than henly, whereas the augmentation of testis by ovary raises the demand of the gonadic tissues upon the body, and this, possibly working through the medium of the thyroid, as previously suggested by one of us (Crew, 1925), is reflected in the change in plumage characterisation. Experiments now being undertaken by the present writers are demonstrating that it is possible to get henly-feathering in the normally cocky-feathered male by augmenting the bird's own testes by abundant testis grafts from other and normally cocky-feathered sources.

It is reasonable to assume, therefore, that the hen now being described became cocky-feathered because in her body was functional testicular tissue, and that she then became henly-feathered because at the time of the development of the second adult plumage there was more active gonadic tissue, mainly, if not wholly, testicular in the body, than is usual in the bird (male and female) that assumes cocky-feathering. No appeal to a legitimate endocrine hypothesis can find accommodation for all the facts.

The expenses incurred during the course of this study were met out of a grant made to one of us by the Government Grants Committee of the Royal Society. For this we wish to express our thanks.

Summary.

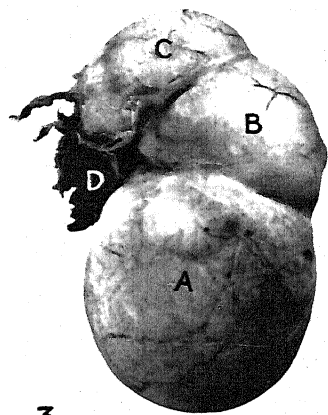
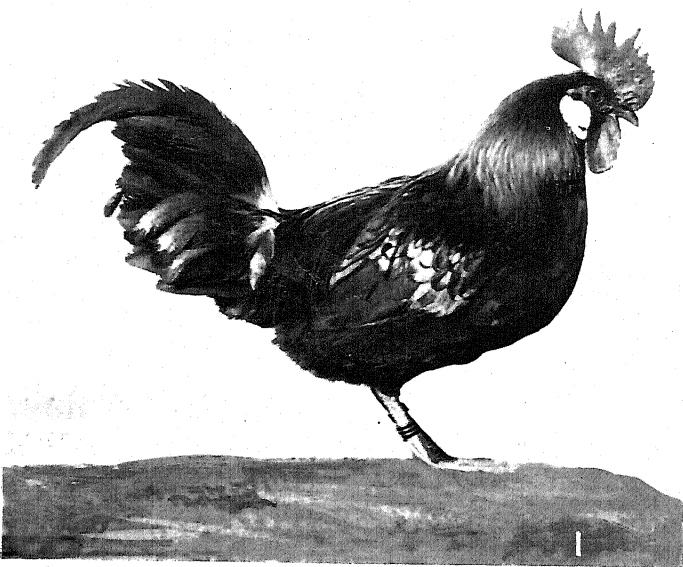
1. Into a Brown Leghorn hen, ovariectomised when four days old, the testes of a brother were implanted. The bird first assumed the plumage characterisation of the cock, but after a general moult her plumage became as that of a hen. Post-mortem examination revealed a small fragment of degenerate ovarian tissue left behind at the time of operation, an active right gonad of testicular structure, and abundant testicular tissue which had developed from the grafts.

2. The situation is discussed and reasons given for holding the opinion that ovarian and testicular tissues in respect of their own individuation exert demands upon the general economy of the same kind, but different in degree; that the functioning of an ovary is physiologically more expensive than is that of the testes; and that it is possible to augment the demands of the testes until they become equivalent to the ovary.

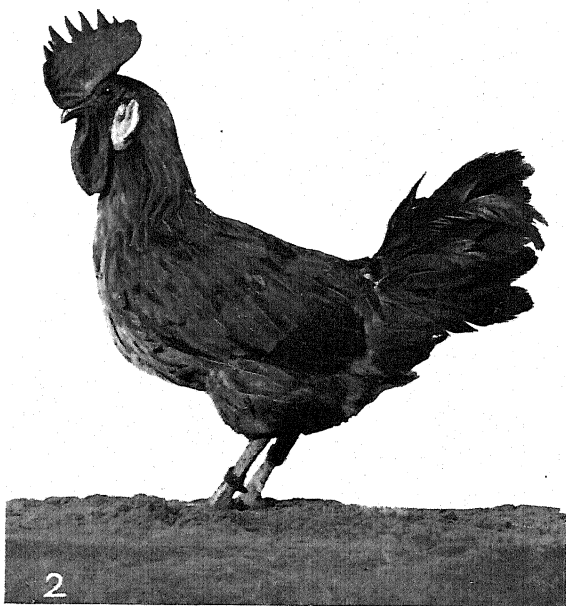
3. It is suggested that this hen became cocky-feathered because in her body at this time there was active testicular tissue, and that she later became henney-feathered because the amount of testicular tissue had become greatly increased and had exerted demands upon the body equivalent to those of an active ovary.

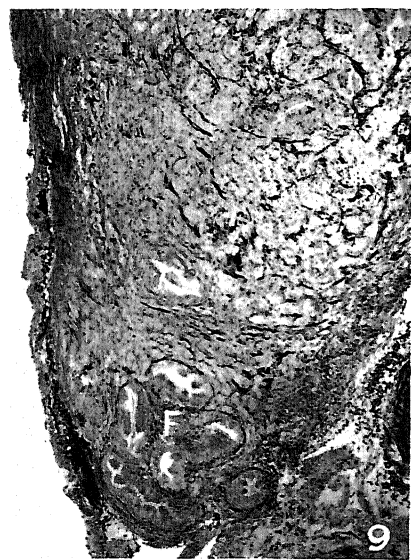
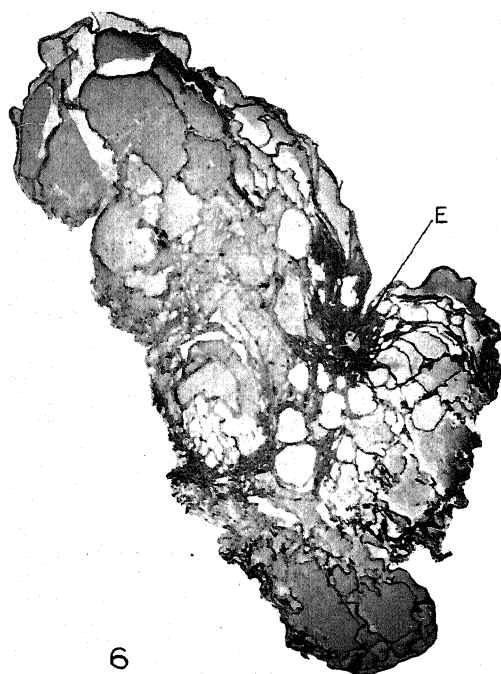
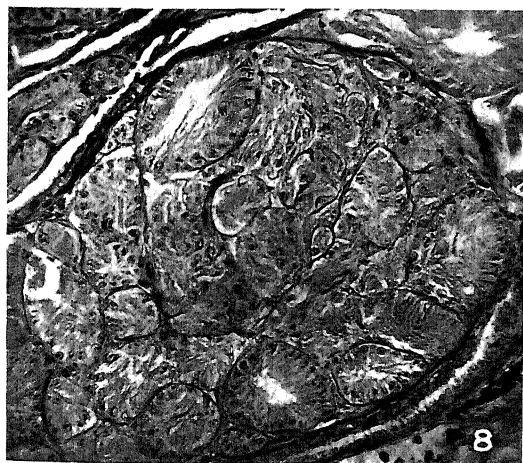
REFERENCES.

- Benoit, J. (1923), "A propos du changement expérimental de sexe par ovariectomie chez la poule," 'C. R. Soc. Biol.,' vol. 89, pp. 1326-1328.
- Benoit, J. (1923), "Transformation expérimentale du sexe par ovariectomie précoce chez la poule," 'C. R. Acad. Sci.,' vol. 177, pp. 1074-1075.
- Crew, F. A. E. (1925), "Rejuvenation of the Aged Fowl through Thyroid Medication," 'Proc. Roy. Soc. Edin.,' vol. 45, pp. 252-260.
- Domm, L. V. (1924), "Sex Reversal following Ovariectomy in the Fowl," 'Proc. Soc. Exp. Biol. and Med.,' vol. 22, pp. 256-278.
- Finlay, G. F. (1925), "Studies on Sex Differentiation in Fowls," 'Brit. Journ. Exp. Biol.,' vol. 2, pp. 439-468.
- Greenwood, A. W. (1925), "Gonad Grafts in the Fowl," 'Brit. Journ. Exp. Biol.,' vol. 2, pp. 469-492.
- Pézar, A. (1918), 'Le conditionnement physiologique des caractères sexuels secondaires chez les oiseaux,' Thesis, Paris.
- Riddle, O. (1925), "On the Sexuality of the Right Ovary of Birds," 'Anat. Rec.,' vol. 30, pp. 365-382.
- Smith, Geoffrey (1910), "Studies in the Experimental Analysis of Sex," 'Quart. Journ. Micr. Sci.,' vol. 55, pp. 225-240.
- Thomson, J. A., and Geddes, P. (1889), 'The Evolution of Sex,' Scott, London, 322 pp.
- Zavadovsky, M. (1922), 'Sex and the Development of its Characters,' State Edit., Moscow, 256 pp.



3.





DESCRIPTION OF PLATES.

PLATE 3.

- FIG. 1.—Photographed at 12 months. The developing spurs are not seen because of the unsatisfactory pose of the bird.
 FIG. 2.—Photographed at 17 months. The spiral band removed from the right leg in order to show the spur.
 FIG. 3.—Left testis graft (natural size).
 FIG. 4.—Section of left testis graft at B (fig. 3) showing tubules in active spermatogenesis. $\times 50$.

PLATE 4.

- FIG. 5.—Section of left testis graft at C (fig. 3) showing large cysts filled with sperm and tubules in active spermatogenesis. $\times 50$.
 FIG. 6.—Section of ovarian fragment at D (fig. 3) showing abundant cystic follicles. $\times 15$.
 FIG. 7.—Higher magnification of previous section at E (fig. 6) to show an occasional normal follicle. $\times 190$.
 FIG. 8.—Section of right gonad showing localised area of atrophic tubules. $\times 190$.
 FIG. 9.—Section of right gonad. At F are seen par-ovarian tubules. The bulk of the section consists of sex cord tissue. $\times 55$.

The Induction of Melanism in the Lepidoptera and its Subsequent Inheritance.

By J. W. HESLOP HARRISON, D.Sc., and F. C. GARRETT, D.Sc.

(Communicated by Prof. E. W. MacBride, F.R.S.—Received November 27, 1925.)

I.—*Introductory.*

The question of melanism in the Lepidoptera is of paramount importance inasmuch as this is one of the few recognizable evolutionary changes which have occurred in Nature in recent times and, for the most part, before the eyes of the present generation.

Moreover, the change is not slight. Wherever melanism arises we see large numbers of species in groups widely separated systematically, developing forms in which white, ochreous and similar ground colours are replaced by black and blackish browns. Once initiated in a species, this transformation is progressive and often exceedingly rapid; frequently the whole of the individuals of the affected species become melanic in an incredibly short period. For example, in 1898 the Geometrid moth, *Ypsipetes (Larentia) trifasciata*, was

quite typical everywhere in North Durham, yet, in 1910, every specimen captured or bred from wild pupæ—and over four hundred were examined—in that district belonged to the black variety, *nigerrima* Harrison; and this is typical of many similar cases.

Furthermore, the necessity for investigating the origin and spread of melanism becomes still more imperative when one recognises that its incidence is, in some way or other, bound up with the industrialisation of the affected area. Of course, admitting as it does the possibility of the Lamarckian factor in evolution, this connection is minimised by those rejecting Lamarckian views, but every worker who approaches the problem in the field ends by emphasizing it. Hasebroek (1925), for instance, gives one of his papers the title “Die prinzipielle Loesung des Problems des Grossstadt- und Industriemelanismus der Schmetterlinge”; Ule (1925) says “Es besteht ein Zusammenhang mit Industriel-gebieten,”* and even Doncaster (1906) writes: “The fact that in so many cases the progress of the change has taken place in, or near, manufacturing centres naturally suggests a relation of cause and effect,” and the circumstance that, “after their appearance on the Continent, these forms have especially established themselves in the neighbourhood of Crefeld, the ‘Black Country’ of Germany, supports the same conclusion.”

Lower down, however, the same writer continues, “In several cases melanics have become prevalent in quite rural localities.” In the latter case, Doncaster clearly had in mind apparently isolated centres of melanism, such as that near Watlingbury, Kent. But even this case falls into the same category as the others, for the woods in which the Kentish melanic forms of *Tephrosia conssonaria* and *Boarmia consortaria* originated receive huge clouds of smoke from the Medway cement works, and display to the full the characteristics of a wood, whether on Tyneside or elsewhere, subjected to smoke action. Personal examination of these woods has satisfied us that lichens have vanished, or are reduced to the immature granulose condition, whilst liverworts and mosses are decadent.

It seems unnecessary to emphasize here the conditions obtaining in melanic areas generally, for that has already been done by one of us in a former paper (Harrison, 1920). In the same publication it was urged that the presence of metallic salts, contaminating the food plants in manufacturing and urban

* The same writer, discussing the black variety *carbonaria* (*doubledayaria*) of *Amphidasys betularia*, very significantly remarks, “Es erfolge keine Einwanderung von England auf den Kontinent; *A. betularia* L. f. *carbonaria* Jord. ist vielmehr selbständig auf dem Festland entstanden.”

areas, might not only in itself affect the germ plasm, so as to produce heritable melanism, but in addition might stimulate it, when once induced, to its maximum expression.

If such be indeed the exciting agents it seemed quite easy to devise decisive experiments. Two distinct lines of attack lie open : (1) Non-melanic strains of lepidopterous insects could be brought from unaffected into melanic areas and there fed on the local food plants ; or (2) food-plants could be artificially charged with certain of the metallic constituents of factory smoke, arbitrarily chosen, known to be of frequent occurrence in or on contaminated foliage, and insects fed on such food in non-melanic regions. Both of these methods were adopted, the salts selected being lead nitrate and manganous sulphate.

The former salt was chosen, because it is well known that lead, in small doses, has a great physiological effect, and the latter because the numerous oxides of manganese are so readily transposed by oxidation and reduction that its reactions are peculiar. Moreover, in the case of manganese, direct estimation of the manganese content of hawthorn leaves (upon which so many of the melanic insects of the Tyne valley feed) shows a progressive diminution as we pass westward from Newcastle-upon-Tyne.

When cut twigs of hawthorn are placed in a dilute solution of a metallic salt the latter is readily and quickly absorbed. As a rule, three parts of the salt in a thousand of water will not cause plasmolysis ; but it was soon discovered that in warm weather, when transpiration was rapid, with manganese salts a brown deposit—probably one of the oxides—forms in the tissues of the leaves, the foliage becoming hard and not readily eaten by larvæ. A solution of one gram per litre does not produce this effect, and sprigs of hawthorn with their cut ends immersed in it retain their freshness about as well as in pure water. Solutions of this strength were, therefore, used throughout these experiments.

It is, of course, essential that the whole of the food should contain the salt, and the speed with which the twigs become charged had to be determined. It was found that when the cut end of a twig, about 40 cms. long, was placed in a solution of lead nitrate (one gram per litre) the lead could be found in the upper portion in about 24 hours. The rule adopted, therefore, was to treat the food for about twenty-four hours before use, and to place the solution in the vessels of the cages in which the larvæ were fed.

The next problem was the choice of suitable insects for our purpose ; not all lepidoptera are suitable, and for diverse reasons. The ideal insect must pass several tests ; it must (1) be easy to rear in captivity, (2) be bivoltine, (3) hiber-

nate as ova or pupæ, and (4) its food plant must be easy to obtain and to keep fresh in a cut state.

These requirements are satisfied by *Selenia bilunaria*, Esper, and *Tephrosia* (*Ectropis*) *bistortata*, Goeze, both of which had been employed by one of us in previous work. These two species therefore supplied our raw material, although *T. crepuscularia*, Bkh., was also used in the preliminary experiments.

Of these three species—all Geometridæ—*Selenia bilunaria* (the Early Thorn) is quite common in the British Isles, and collectors take or rear great numbers every season, but no melanic form has ever been taken wild; it appears liable to remarkably slight variation, other than that differentiating the summer brood, *juliaria*, Haw., from the typical spring brood. On the other hand, melanic forms of *Tephrosia bistortata* (the Engrailed) have been reported from Wales, but not from England or Scotland. In the case of its congener *T. crepuscularia* (the Small Engrailed) melanic forms are prevalent in many industrial areas in England and Wales, and on the Continent. In our district (South Northumberland and North Durham) neither *crepuscularia* nor *bistortata* is melanic, nor does either approach very closely to the smoke zone; further, both are single-brooded here. But, and this is very important, wherever closely-allied forms, such as *Boarmia repandata* L. and *B. gemmaria*, Brh., reach the Tyne, they are quite black, and in South Durham, where *T. crepuscularia* comes within the sphere of influence of Dinsdale Iron Works, its melanic form *delamerensis* B.W. appears.

II.—The Experiments.

We began with *Selenia bilunaria* from Kent and Sussex, *Tephrosia bistortata* from Kent, Hampshire and North Yorks, and *T. crepuscularia* from Kent, some being reared at Birtley, a district producing great numbers of melanic forms, and others at Hexham, where melanism occurs to a slight extent, and is of the infiltrated order. The treatment accorded to the individual broods will be stated as each is discussed.

Early Experiments.—Before the main experiments were undertaken certain preliminary ones had been carried out, and these we shall describe briefly. These commenced with insects of typical strains of *Selenia bilunaria* from the south, fed on wild plants at Birtley in 1918. At that time there were in Birtley, in addition to its usual industries (coal mines, iron and brick works), enormous munition works which poured forth, day and night, huge clouds of smoke containing copper, zinc, arsenic and other substances. In consequence, all foliage was contaminated to an unprecedented degree, and so serious were

the effects, that allotment holders took concerted action to get the nuisance diminished.

Under such conditions, and on food gathered by the roadside, were the progeny of the southern moths reared for two generations. In 1919, the spring brood consisted of 84 types, five insects which could be regarded as melanochoiric, and two which were uniformly leaden black. One of these black moths was mated with an unrelated typical male, of Welsh origin, and fertile eggs obtained. The resulting larvæ were fed on hawthorn taken from isolated trees growing in a large oak wood, and 90 moths (52 ♀ ♀ and 38 ♂ ♂) emerged, all perfectly typical.

Two of these were paired, and their offspring reared, but the period of *éclosion* proved to cover, unfortunately for the continuation of the experiments, the months of September, October, November and December. This brood contained 27 type insects and 7 melanics, suggesting that the melanism was behaving as a Mendelian recessive.

Similarly, in 1920, a strain of *Tephrosia crepuscularia*, imported from Kent in 1918, reared on the same bushes as in the case of *S. bilunaria*, yielded, in a small brood of 23 moths, a single black female. This was paired with an ordinary male of Staffordshire parentage, and in 1921 an F₇ generation of 21 insects, all black, appeared. In consequence of the long duration of the period of emergence, no pairing was obtained, and for this reason, and because of difficulty in rearing this species, its use was abandoned. It was clear, however, that the melanism was inherited as a Mendelian dominant, precisely as in the case of the similar form which is found wild.

THE MAIN EXPERIMENTS.

(A) *With Selenia bilunaria*, Esp.

In this series we began with a small batch of eggs laid by a wild Abbot's Wood (Sussex) female, captured in July, 1921. The larvæ from these hatched in August, and as we contemplated critical work in several directions, particular care was taken with their food, this being procured in the oak wood* already mentioned. Nearly 70 pupæ were obtained, and 59 imagines (26 ♀ ♀ and 33 ♂ ♂) emerged in 1922. This batch was, except for the normal sexual dimorphism, of a very uniform composition, differing in no wise from other spring broods in our possession which had originated in various southern habitats. Two moths chosen at random from these were mated to continue the strain, and the larvæ

* The species in this wood are remarkable for their typical facies, *Phigalia pedaria* and *Oporabia dilutata* being especially noteworthy in this respect.

obtained were fed from the same pure hawthorn as before; these produced moths in June and July. This brood, with the exception of five insects, we still possess; it consists of 24 ♀ ♀ and 35 ♂ ♂, manifesting a very small range of variation, the colour tendencies leaning perhaps to the lighter side. The moths can only be classified as very ordinary specimens of the summer form *juliaria*.

Pairings were made, and two small batches, each of about 60 ova, from different females, were sent to Hexham to be reared there. One of these batches was divided, one half being destined to serve as a control, while the larvæ from the other half were reared on food (hawthorn) to which lead nitrate had been added by the method already described. The other batch was divided similarly, half providing a control, while the other was used for an experiment with manganese sulphate. The hawthorn on which all four groups were fed was cut from a particular tree, known from direct analyses to contain an unusually small amount of manganese.

We shall record the history of the lead experiments first. Concerning the controls, it is only necessary to say that three successive inbred generations were fed on untreated food, and to emphasise the fact that they contained nothing but typical insects. The latter held true of the brothers and sisters of the first of these generations which had had lead in their diet. Emerging in the spring of 1923, they showed no special variation, and resembled in every way the spring brood of the previous year reared at Birtley. But the summer broods of 1923, inbred from the spring broods of that year, and continued on a diet containing lead, showed a marked difference. Two such families, each from about 60 eggs, were reared, exactly as before, and moths secured. The first (family 1923 A L*) included 15 ♀ ♀ and 12 ♂ ♂, but of these one male was practically black, whilst the remainder were ordinary *juliaria* insects.

Similarly, the companion brood (1923 B L) gave 11 ♀ ♀ and 20 ♂ ♂, again all typical except for two black males which resembled that from 1923 A L. Attention is specially directed to the absence of any figures offering scope for the suggestion that these melanics are due to the segregation of a Mendelian recessive character. The disparity between ratios of 26:1 and 29:2, on the one hand, and 3:1 on the other, is too great for that, independently of the absence of all trace of melanism in the controls.

Proceeding, a type female of 1923 A L was paired with a type male of 1923 B L, and the resulting larvæ reared on hawthorn with lead nitrate in it.

* The second capital following the year of the brood is to designate the origin of the melanism, "L" being used when it owed its induction to lead, and "M" to manganese.

As a result, a batch of moths (1923 L L) was obtained, including 7 ♀ ♀ and 12 ♂ ♂, two females and one male being melanic. Once more the departure of a ratio of 17 : 3 from that of 3 : 1 is too great to be interpreted as a chance deviation, and again there is no suggestion of the reappearance of a Mendelian recessive.

Work with 1923 L L did not end here, for two of the type moths were paired, and their offspring reared on untreated hawthorn. The brood (1923 T L) consisted of 23 ♀ ♀ and 18 ♂ ♂, all of the type form. The inbreeding was continued, and another brood (1924 L L) reared on pure food; the resulting 24 moths were all types, proving that at least one of their parents and one of their grandparents had been free from recessive melanism.

Melanism having appeared, it was necessary to determine whether it was inherited or not, and this was done. The melanic male of 1923 A L was mated with a light female of 1923 B L, and the larvæ reared on untreated hawthorn. The F₁ generation (1923 K L) consisted of 11 ♀ ♀ and 15 ♂ ♂, all types, thereby demonstrating that the melanism, if inherited at all, was behaving as a Mendelian recessive. Inbreeding these F₁ insects, and continuing to supply untreated food, gave us three F₂ families—1923 S L, 1924 F L, and 1924 J L. The first-named gave 8 types and 2 melanics, the second 13 types and 4 melanics, and the third 49 types and 17 melanics. Adding these F₂ families together, we get a total of 70 types and 23 blacks—a ratio of 3·04 : 1, which harmonises well with the 3 : 1 expected if we are dealing with an ordinary Mendelian recessive. Further, this ratio should be contrasted with those for the broods containing the original melanics.

Let us now consider the outcome of breeding from the melanics developed in the parallel family 1923 L L, which had received lead in its food after its administration to the others had ceased. Here a new feature was introduced into the experiment, in the form of typical insects bred from wild Bohemian larvæ; of these a type female was paired with a black male of 1923 L L. The F₁ brood so produced, 1924 C L, included nothing but types, 140 in number, and two of these were mated to secure the critical F₂ brood. This gave us the large F₂ family 1924 R L, consisting of 144 types and 41 melanics.

Again coupling these with the composition of the F₁ brood, 1924 C L, we have incontrovertible evidence that the melanism has been inherited as a simple recessive; in other words, we have ample confirmation of the evidence yielded by the line bred from 1923 K L.

One of the black females of 1923 L L was paired with a typical brother, giving the brood 1923 V L; this comprised 24 ♀ ♀ and 15 ♂ ♂, all quite typical; but,

as the moths emerged in December, 1923, and January, 1924, they were useless for continuing the investigation.

In conjunction with the manganese brood 1924 S M, there was abundant scope to investigate the results of mating black moths *inter se*. Two such pairings were made, one between a black female from the brood 1924 S M and a black male from 1924 R L, and the other an inbreeding of black insects of the latter parentage. In each case the resulting family was large, that (1925 A L M) from the first-named giving 59 ♀ ♀ and 66 ♂ ♂, all black, and the other (1925 B L) 36 ♀ ♀ and 39 ♂ ♂, all black except two males. These two unexpected males, in view of the state of all the other broods reared, can only be explained on the assumption of an accidental introduction of two eggs or larvæ from another batch, or of "reverse" mutation similar to that to which reference is made in recounting certain *Drosophila* work (Morgan, Bridges, Sturtevant, 1925).

The work with this line did not end here, for two black moths from 1925 A L M were paired and their offspring (1925 D L M) successfully reared. Most of the pupæ are lying over the winter, but five moths have emerged in the autumn of the present year, and all are black. Similarly a pair from 25 B L have given 1925 E L, of which one black male has come out, the remainder still being pupæ.

Turning now to the manganese work, we find the controls were a failure, for in the first generation only two moths, both typical males, appeared. On the other hand, the treated insects did well, and in the second such generation 1923 D M gave (like the corresponding broods of the lead series) melanics, the batch of 9 ♀ ♀ and 11 ♂ ♂, including two black ♀ ♀ and six black ♂ ♂. Moreover, typical insects were practically unrepresented, for the insects not fully melanic were, in varying degrees, melanochoic.

To continue this series, the desired mating between two melanics proving unobtainable, owing to the failure of such insects to synchronise in emergence, the other two possible combinations were made. One of these involved mating a black male with one of its more or less melanochoic sisters, and the other the mating of a second black male with a female from the control family 1923 C used in the lead experiments. Fertile ova were laid, larvæ appeared, and these were fed, like all subsequent broods, on pure food. The history of the inbred pair will be taken first.

The insects bred (1923 O M) were 29 in number, including 14 ♀ ♀ and 15 ♂ ♂, of which 5 ♀ ♀ and 7 ♂ ♂ were black. Thus the brood comprised 17 types and 12 melanics—a figure indicating (1) that again melanism was behaving

as a Mendelian recessive, and (2) that the second parent was heterozygous for that character.

Work with this brood was continued, and three distinct types of mating were brought about, one between two black moths producing family 1924 E M, a second between two types resulting in 1924 I M, and a third between a type male and a Bohemian female giving 1924 H M. Of these 1924 E M contained 13 moths, according to expectation, all black; 1924 I M was composed of 59 types and 14 blacks, and 1924 H M of 40 typical insects. Without exception, these results confirm our view that, not only is the induced melanism inherited, but inherited as a Mendelian recessive, for clearly the figures in 1924 I M are a chance departure from a 3 : 1 ratio.

Continuing, we paired a black male of 1924 I M with a type female from 1924 C L known to be heterozygous for black, and obtained a brood 1924 S L M composed of 46 typical and 35 black insects. Taking a female from the heterozygous type insects of this family, and mating her with one of the black males, we obtained a batch (1925 J W T) consisting of 30 melanics and 30 types. Both of these results agreed with our anticipations.

The course of events with the F_1 family (1923 P M) bred when the black male was crossed with a type control female, was much the same. Twenty-five typical insects (15 ♀ ♀ and 10 ♂ ♂ appeared); two matings of these moths were made *inter se*, and a typical male was outcrossed with a Bohemian female. The last-named gave a batch (1924 G M) of 72 insects, and as was expected none were melanic. The two other pairings, however (inbred) gave the F_2 batches 1924 A M and 1924 B M, both containing melanics as well as types, the former being composed of 40 types and 12 blacks, and the latter of 50 types and 15 blacks. Pairing two of the blacks of 1924 B M we obtained, finally the small, but significant, family 1924 Q M—8 black and no types.

Table I.—Showing *Selenia bilunaria* families in which induced melanism appeared.

Family.	Treatment.	Females.		Males.		Remarks.
		Types.	Melanics.	Types.	Melanics.	
23 AL	Lead	15	—	11	1	—
23 BL	Lead	11	—	18	2	—
23 LL	Lead	6	2	11	1	Ex type 23 A ♀ × type 23 B ♂.
23 DM	Manganese....	7	2	5	6	The insects classed as types are more or less melanochoic.

Table II.—Homozygous type \times homozygous melanic.
 $TT \times tt$.

Family.	Origin of Family.	Types.	Melanics.
23 KL	23 B L ♀ \times 23 A L ♂	26	0
23 PM	Control ♀ \times 23 D M ♂	25	0
23 VL	23 L L ♀ \times 23 L L ♂	39	0
24 CL	Bohemian ♀ \times 23 L L ♂	140	0
	Actually reared	230	0
	Theoretical result	230	0

Table III.—Heterozygous type \times homozygous black.
 $Tt \times tt$.

Family.	Origin of Family.	Types.	Melanics.
23 OM	23 D M ♀ \times 23 D M ♂	17	12
24 SLM	34 CL ♀ \times 24 I M ♂	46	35
25 J.W.T.*LM	24 SLM ♀ \times 24 SLM ♂	30	30
	Actual result	93	77
	Theoretical result....	85	85

* The J.W.T. indicates that this brood was reared at Holywell, Northumberland, by Mr. J. W. Thompson.

Table IV.—Heterozygous type \times heterozygous type.
 $Tt \times Tt$.

Family.	Origin of Family.	Types.	Melanics.
23 SL	23 K L ♀ \times 23 K L ♂	8	2
24 AM	23 P M ♀ \times 23 P M ♂	40	12
24 BM	23 P M ♀ \times 23 P M ♂	50	15
24 FL	23 K L ♀ \times 23 K L ♂	13	4
24 IM	23 O M ♀ \times 23 O M ♂	59	14
24 JL	23 K L ♀ \times 23 K L ♂	49	17
24 RL	24 C L ♀ \times 24 C L ♂	144	41
	Actually reared	363	105
	Theoretical result	351	117

Table V.—Homozygous black \times homozygous black.

$tt \times tt$.

Family.	Origin of Family.	Types.	Melanics.
24 EM	23 O M ♀ \times 23 O M ♂	0	13
24 QM	24 B M ♀ \times 24 B M ♂	0	8
25 ALM	24 S M ♀ \times 24 R L ♂	0	125
25 BL	24 R L ♀ \times 24 R L ♂	2*	75
25 DLM†	25 A L M ♀ \times 25 A L M ♂	0	5
25 EL†	25 B L ♀ \times 25 B L ♂	0	1
	Actual result	2	227
	Theoretical result	0	229

* Probably accidental introductions from another family.

† Brood still emerging.

Table VI.—Homozygous type \times heterozygous type.

$TT \times Tt$.

Family.	Origin of Family.	Types.	Melanics.
24 GM	Bohemian ♀ \times 23 P M ♂	72	0
24 HM	Bohemian ♀ \times 23 O M ♂	60	0
	Actual result	132	0
	Theoretical result	132	0

Table VII.—Either extracted homozygous type \times heterozygous type or extracted homozygous type \times extracted homozygous type.

$TT \times Tt$

or $TT \times TT$.

Family.	Origin of Family.	Types.	Melanics.
24 KL	23 SL ♀ \times 23 SL ♂	22	0
24 ML	23 SL ♀ \times 23 SL ♂	5	0
	Actual result	27	0
	Theoretical result	27	0

Taking the whole of these experiments with both lead and manganese, and including also those in the preliminary work, only one conclusion is possible—that the melanism induced in our experiments is inherited as a Mendelian recessive.

(B) With *Tephrosia bistortata* Goeze.

The work with this species commenced with two unrelated typical strains brought from Kent and Hampshire respectively, each of which formed the raw material for a distinct experiment. The former came into our hands in spring, 1921, in the form of a batch of eggs deposited by a wild type female, which was also sent with them. The Hampshire material reached us a little earlier the same year, as pupæ reared indoors in Hampshire from ova laid by a wild typical summer brood female of the preceding year.

Although, for certain reasons which will be developed subsequently, we propose to discuss these experiments separately, the treatment of the various broods was much the same in both cases. Both pieces of work were carried out at Birtley, N. Durham, and the whole of the experimental larvæ, in the initial stages, were fed on hawthorn gathered on the roadside in a station where melanism abounds. On that roadside, all of the representatives of certain species are either melanic or melanochoic, whilst, in the case of others, the type still persists in company with an abundance of melanic or melanochoic forms; amongst the former are *Boarmia repandata*, *B. gemmaria*, *Amphidasys betularia*, *Oporabia dilutata*, *Ypsipetes sordidata*, and *Y. trifasciata*, and amongst the latter *Polia chi*, *Xylophasia monoglypha*, *Cheimobacche fagella* and *Dasystoma salicella*.

Presumably, if such food as these insects ate in the larval condition, or certain chemical compounds in it, were responsible for the genesis of natural melanism, one would anticipate that if given to non-melanic strains brought from unaffected areas for a series of generations, in the end melanic forms should appear. These opinions were submitted to experimental proof in the work we shall now describe.

The History of the Kentish Strain.—The batch of eggs received (a fairly large one) was divided into two approximately equal lots. The first of these, to be regarded as controls, yielded larvæ, which were fed on newly expanded oak leaves, replaced in June by seedling knot-grass (*Polygonum aviculare*) grown in the garden. The experimental batch, on the other hand, received the roadside hawthorn mentioned above.

Both sets emerged freely as imagines in June and no differences in appearance were discernible, each lot closely resembling the other and manifesting the usual fluctuating variation, of small range, proper to the summer brood of *T. bistortata*. In both lots, influenced no doubt by the great heat of the summer of 1921, a third brood, resembling the second in every way, appeared; the controls of this generation were again provided as larvæ with knot-grass, and the others with hawthorn.

From the oak knot-grass series, a pair was chosen, and larvæ obtained, to be fed as before. These produced typical imagines in the spring of 1922. Again, the same inbreeding was continued, and the same feeding methods adopted, the May-June larvæ receiving oak and knot-grass, and the August lot knot-grass alone. This procedure was maintained year by year until March, 1925, when, as a pure line, the strain ran out.

Throughout the period of five years, representing ten generations, during which this control lot was kept going, although certain portions of it were regularly inbred, and in addition outcrossed repeatedly in experimental work with *T. crepuscularia*, and with induced melanic *bistortata*, never at any stage did it display the faintest indications of carrying recessive melanism.

Much the same held true during 1922 with the parallel batches raised from the half brood started on roadside hawthorn; no melanism arose. However, in 1923, the fifth inbred generation (family 23a) reared with such a diet, a large one composed of 97 insects (43 ♀ ♀ and 54 ♂ ♂), included, with 96 typical insects, one black female.

This insect, except for the white subterminal line or band, characterising so many melanic Geometridæ, can only be described as being coal-black, and, as a matter of fact, when fresh it could only with difficulty be differentiated from the melanic forms of the closely allied *Tephrosia consonaria* and *Boarmia repandata*.

In order to determine whether this melanism was heritable or not she was mated to a typical brother, and eggs duly secured. The resulting F₁ lot were transferred to an oak knot-grass regimen, and imagines came out in late June in the form of 77 type insects—31 ♀ ♀ and 46 ♂ ♂.

Precisely as with *Selenia bilunaria*, the melanism, if inherited, was showing itself to be a Mendelian recessive.

Out of this F₁ lot, two pairs were caged up to give two batches of eggs, followed by larvæ which were likewise provided with knot-grass. The two broods behaved rather differently, the moths of the first F₂ lot (family 23s) emerging in two relays, one in August and September, 1923, and the second in March, 1924, while the whole of the other F₂ batch (24a) put in an appearance in spring, 1924. The composition of these broods in respect to colour and sex is set out in Table IX.

Of the section of 23s, reared in September, 1923, a typical male was mated with a melanic female, and progeny obtained. Thus, there were coming out in the breeding pots in spring, 1924, three families containing black insects, two of the F₂ series and one F₃ family (24b).

Utilising (1) these black insects, (2) their typical brothers and sisters, (3) the

typical control families, as well as (4) a typical strain of *T. crepuscularia*, the necessary combinations for pursuing our inquiry into the inheritance of the melanism were made. From all these matings eggs were secured, and the broods arising therefrom, fed on knot-grass, successfully reared to the imaginal condition.

Tables X–XV serve to reveal the make-up of these broods in the critical matters of sex and colour.

The same course was pursued with the succeeding summer brood of 1924 and with the spring and second* broods of 1925. As the types of mating in these last three generations, in respect to melanism, are exactly the same as those brought about amongst the second half of the F_2 lot 23s, the F_2 brood 24a, and the F_3 brood 24b, the results, with all the pertinent facts, are given in the same series of tables.

Once more every scrap of evidence these tables have to offer points uniformly and clearly in the same direction, i.e., to the conclusion that here the induced melanism is inherited as a Mendelian recessive.

Table VIII.— F_1 cross of the original melanic *T. bistortata* ♀ with a type ♂.
 $tt \times TT$.

Family.	Origin of Family.	Types.			Melanics.		
		Females.	Males.	Total.	Females.	Males.	Total.
23f	Fifth inbred generation from Kentish stock obtained in 1921	31	46	77	—	—	0

Table IX.— F_2 lots from Family 23f.
 $Tt \times Tt$.

Family.	Origin of Family.	Types.			Melanics.		
		Females.	Males.	Total.	Females.	Males.	Total.
23s	23f ♀ × 23f ♂	42	41	83	12	16	28
24a	23f ♀ × 23f ♂	25	28	53	8	11	19
	Actual result			136			47
	Theoretical result			137.25			45.75

* The offspring from this, instead of lying over until March, 1926, emerged as a third brood this year.

Table X.—Homozygous (or Heterozygous) type \times homozygous type.
 TT (or Tt) \times TT .

Family.	Origin of Family.	Types.			Melanics.		
		Females.	Males.	Total.	Females.	Males.	Total.
24e	24a ♀ \times 23s ♂	23	18	41	—	—	0
24h	23s ♀ \times 24a ♂	28	29	57	—	—	0
25x	25p ♀ \times 25q ♂	12	14	26	—	—	0
	Actual result			124			0
	Theoretical result			124			0

Table XI.—Homozygous type \times heterozygous type.
 $TT \times Tt$.

Family.	Origin of Family.	Types.			Melanics.		
		Females.	Males.	Total.	Females.	Males.	Total.
24f	24b ♀ \times control ♂	47	49	96	—	—	0
24g	Control ♀ \times 24b ♂	28	31	59	—	—	0
24i	24b ♀ \times 23s ♂	26	32	58	—	—	0
25l	24p ♀ \times 24g ♂	32	34	66	—	—	0
	Actual result			279			0
	Theoretical result			279			0

Table XII.—Heterozygous type \times heterozygous type.
 $Tt \times Tt$.

Family.	Origin of Family.	Types.			Melanics.		
		Females.	Males.	Total.	Females.	Males.	Total.
25c	24n ♀ \times 24n ♂	20	26	46	8	7	15
25d	24n ♀ \times 24k ♂	19	22	41	6	8	14
25e	24l ♀ \times 24l ♂	33	30	63	7	11	18
25p	25h ♀ \times 25d ♂	8	10	18	1	5	6
25q	25e ♀ \times 25i ♂	32	40	72	12	11	23
25v	25r ♀ \times 25s ♂	28	24	52	8	9	17
25w	25p ♀ \times 25s ♂	13	15	28	6	4	10
	Actual result			320			103
	Theoretical result			317.25			105.75

Table XIII.—Homozygous black \times homozygous black.
 $tt \times tt$.

Family.	Origin of Family.	Types.			Melanics.		
		Females.	Males.	Total.	Females.	Males.	Total.
24c	24b ♀ \times 24a ♂	—	—	0	23	31	54
24d	24a ♀ \times 23s ♂	—	—	0	17	22	39
25a	24d ♀ \times 24c ♂	—	—	0	23	21	44
25b	24k ♀ \times 24d ♂	—	—	0	32	34	66
25m	25h ♀ \times 25i ♂	—	—	0	17	18	35
25n	25b ♀ \times 25h ♂	—	—	0	9	8	17
25o	25e ♀ \times 25i ♂	—	—	0	22	28	50
	Actual result			0			305
	Theoretical result			0			305

Table XIV.—Homozygous black \times homozygous type.
 $tt \times TT$.

Family.	Origin of Family.	Types.			Melanics.		
		Females.	Males.	Total.	Females.	Males.	Total.
24p	Control type ♀ \times bl. 24b ♂	68	74	142	—	—	0
24m	Bl. 24b ♀ \times type cont. ♂	37	35	72	—	—	0
24n	Bl. 24a ♀ \times type cont. ♂	29	28	57	—	—	0
24l	Type cont. ♀ \times bl. 23s ♂	21	16	37	—	—	0
25k	Bl. 24d ♀ \times type 24g ♂	24	28	52	—	—	0
25f	Bl. 24k ♀ \times type cont. ♂	18	14	32	—	—	0
25g	Bl. 24k ♀ \times type 24e ♂	28	29	57	—	—	0
25s	Type cont. ♀ \times bl. 25a ♂	23	16	39	—	—	0
26	Type 25g ♀ \times bl. 25p ♂	17	20	37	—	—	0
	Actual result			525			0
	Theoretical result			525			0

Table XV.—Homozygous black \times heterozygous type.
 $tt \times Tt$.

Family.	Origin of Family.	Types.			Melanics.		
		Females.	Males.	Total.	Females.	Males.	Total.
24b	Type 23s ♀ \times bl. 23s ♂	19	17	36	18	18	36
24o	Type 24b ♀ \times bl. 24a ♂	14	16	30	12	15	27
24k	Bl. 24a ♀ \times type 24b ♂	23	21	44	22	19	41
25h	Bl. 24c ♀ \times type 24m ♂	16	19	35	18	15	33
25i	Bl. 24d ♀ \times type 24g ♂	14	14	28	18	12	30
25r	Type 25c ♀ \times bl. 25b ♂	8	12	20	13	11	24
25s	Type 25e ♀ \times bl. 25h ♂	21	17	38	12	19	31
25y	Type 25s ♀ \times bl. 25p ♂	13	11	24	12	13	25
25z	Bl. 25p ♀ \times type 25s ♂	15	7	22	9	10	19
	Actual result			277			266
	Theoretical result			271.5			271.5

The Course of Events with the Hampshire Strain.—The moths of the Hampshire strain of *Tephrosia bistortata* can easily be distinguished from those of Kentish origin by their cleaner ground colour. They, like the latter, appeared in three successive broods in the hot season of 1921. In these three broods the spread of variation was very trivial; in fact, even the seasonal dimorphism was at a minimum, the spring brood differing very little indeed from the two summer broods.

In the spring of 1922 difficulty in securing the necessary pairings within the limits of the race rendered it necessary to outcross a male with a female of the univoltine race from Kildale—a dale in the Cleveland Hills, North Yorks.

The eggs obtained from this pairing hatched well, and the larvæ from them were supplied with hawthorn from the same bushes as before. Of this batch 28 ♀♀ and 33 ♂♂ came out in July, 1922, whilst 13 ♀♀ and 17 ♂♂ lay over until 1923. This brood, as well as those from Kildale which preceded it, consisted solely of typical* insects.

Mating up a pair taken from the earlier portion (summer, 1922) we secured a spring brood in 1923, and from that, by in-breeding once more, a summer generation the same year. The March–April brood showed nothing worthy of note. On the other hand, amongst the summer brood insects, we detected a single female with the costal area of the right forewing marked with a broad black stripe, but otherwise perfectly typical. This insect was paired with a type to yield family 24a', reared on knot-grass and including types only.

Continuing, we inbred this family, and obtained families 24b', 24c' and 24d', all of which had oak and garden knot-grass as food; without exception, these three families, as will be determined from Table XVII, consisted of types and melanics† in ratios in complete agreement with Mendelian expectation.

Judging from the composition of these families, we are justified in concluding that the female just described had been, germinally speaking, either homozygous or heterozygous for melanism. Moreover, despite its phenotypical appearance, it is almost certain that the former is the correct interpretation of the facts, for the chances are great indeed against our choosing at random six heterozygous insects from a culture including equal numbers

* Although for years (since 1906) we have been accustomed to observe and rear the Kildale insect in hundreds, never at any time have we seen a melanic *Tephrosia bistortata* from that district; nor, indeed, have our friends Lofthouse, Elgee and Sachse, all of whom have had 25 years' experience with the insect.

† These melanics are identical in appearance with those bred in the preceding series of experiments with Kentish insects.

of homozygous and heterozygous types, as would have been the case had the striped female been a heterozygote for melanism.

With the aid of these F_2 insects and others available we proceeded with our tests, making all the matings we considered capable of throwing further light on the inheritance of the melanism in the *bistortata* cultures, with the result that the facts presented in Tables XVIII-XXII were secured.

Once more it is quite evident that the melanism behaves as a simple Mendelian recessive, and this falls in line absolutely with the circumstances attending the melanism induced in the Kent strain.

Finally, we brought about cross-pairings between insects selected from the Kent families of 1925, and from the mixed Hampshire-Cleveland lots of the same year. As will be perceived from Table XXIII, our views, based on the other sets of experiments, received ample confirmation.

Table XVI.— F_1 ex Hampshire-Cleveland ♀ with black bar on costa × type ♂.

$t t (?) \times T T$.

Family.	Origin of Family.	Types.			Melanics.		
		Females.	Males.	Total.	Females.	Males.	Total.
24a'	Barred ♀ × type ♂	41	47	88	—	—	0

Table XVII.— F_2 generations obtained by inbreeding the F_1 insects set out in Table XVI.

$T t \times T t$.

Family.	Origin of Family.	Types.			Melanics.		
		Females.	Males.	Total.	Females.	Males.	Total.
24b'	24a' ♀ × 24a' ♂	42	44	86	10	14	24
24c'	24a' ♀ × 24a' ♂	16	13	29	4	6	10
24d'	24a' ♀ × 24a' ♂	32	33	65	10	12	22
	Actual result			180			56
	Theoretical result			177			59

Table XVIII.—Later heterozygous types \times heterozygous types.

$Tt \times Tt$.

Family.	Origin of Family.	Types.			Melanics.		
		Females.	Males.	Total.	Females.	Males.	Total.
25k'	24b' ♀ \times 24d' ♂	8	6	14	2	2	4
25l'	25i' ♀ \times 25i' ♂	29	32	61	9	11	20
25m'	25g' ♀ \times 25i' ♂	26	29	55	8	10	18
25n'	25j' ♀ \times 25f' ♂	9	6	15	4	2	6
	Actual result			145			48
	Theoretical result			144.75			48.25

Table XIX.—Homozygous black \times homozygous black.

$tt \times tt$.

Family.	Origin of Family.	Types.			Melanics.		
		Females.	Males.	Total.	Females.	Males.	Total.
25o'	25k' ♀ \times 25h' ♂	—	—	0	8	11	19
25p'	25g' ♀ \times 25f' ♂	—	—	0	17	26	43
25q'	25h' ♀ \times 25k' ♂	—	—	0	8	4	12
25r'	25g' ♀ \times 25g' ♂	—	—	0	17	19	36
	Actual result						110
	Theoretical result						110

Table XX.—Homozygous black \times heterozygous type.

$tt \times Tt$.

Family.	Origin of Family.	Types.			Melanics.		
		Females.	Males.	Total.	Females.	Males.	Total.
25e'	Bl. 24b' ♀ \times type 24c' ♂	18	17	35	17	14	31
25f'	Type 24c' ♀ \times bl. 24d' ♂	7	8	15	4	8	12
25h'	Type 24c' ♀ \times bl. 24c' ♂	12	14	26	16	15	31
25g'	Bl. 24d' ♀ \times type 24b' ♂	18	20	38	14	15	29
	Actual result			114			103
	Theoretical result			108.5			108.5

Table XXI.—Homozygous black \times homozygous type.
 $tt \times TT$.

Family.	Origin of Family.	Types.			Melanics.		
		Females.	Males.	Total.	Females.	Males.	Total.
25i'	Type 24c' \varnothing \times bl. 24b' σ	29	26	55	—	—	0
25j'	Bl. 24c' \varnothing \times type control σ	33	38	71	—	—	0
	Actual result			126			0
	Theoretical result			126			0

Table XXII.—Homozygous type \times homozygous (or heterozygous) type.
 $TT \times TT$ (or Tt).

Family.	Origin of Family.	Types.			Melanics.		
		Females.	Males.	Total.	Females.	Males.	Total.
25s'	24d' \varnothing \times 24c' σ	41	44	85	—	—	0
	Actual result			85			0
	Theoretical result			85			0

Table XXIII.—Miscellaneous intercrossings between the Kent and the Hampshire-Cleveland strains.

Family.	Origin of Family.	Types.			Melanics.			Type of Mating.	Ratio Theoretical Type : Melanic.	Actual Ratio Type : Melanic.
		Females.	Males.	Total.	Females.	Males.	Total.			
25a	Type 25k' \varnothing \times bl. 25c σ	24	27	51	—	—	0	$TT \times tt$	All types	All types
25b	Bl. 25e \varnothing \times type 25i' σ	13	17	30	18	14	32	$tt \times Tt$	1 : 1	1 : 1.06
25g	Type 25c \varnothing \times type 25g' σ	29	25	54	8	9	17	$Tt \times Tt$	3 : 1	3.17 : 1
25h	Type 25f' \varnothing \times type 25d σ	33	40	73	12	11	24	$Tt \times Tt$	3 : 1	3.04 : 1
25e	Type 25k' \varnothing \times type 25c σ	31	29	60	—	—	0	$TT \times TT^*$	All types	All types
25f	Bl. 25k' \varnothing \times type 25c σ	8	7	15	7	9	16	$tt \times Tt$	1 : 1	1 : 1.06

* Or $TT \times Tt$.

III.—*Concluding Remarks.*

We have no intention of entering into any long theoretical disquisition on the subject of our experiments ; the facts are too unmistakable for that.

Making certain deductions from field observations carried out for lengthy periods in many of the most suitable localities, as to the origin of natural melanism, we developed a certain technique to test our views. As a result of both of the possible lines of approach, success attended our efforts ; melanism of the most pronounced type appeared in our cultures. This melanism we are entitled to consider induced by us.

Furthermore, by exhaustive breeding experiments, carried out in all possible directions, we have demonstrated, in all cases with absolute certainty, that this melanism is not only inherited, but inherited as a simple Mendelian unit character.

Our work, therefore, can only be accepted or rejected. If accepted by those wishing to minimise its value, only one objection can be brought against it, and that is the usual one, that in place of a genuine Lamarckian effect we are dealing with a case of parallel induction, soma and germ being equally affected by our operations. We submit that this distinction is merely academic. Whether we are dealing with the induced change of habit, as in Kammerer's work, or with our own, we state without hesitation that the import is plain ; environmental influences have been brought to bear, powerful enough to influence the germ plasm.

If this is once granted we fail to see how, by explaining away any experimental result on the basis of parallel induction, or of direct action on the germ plasm, such as is used to weaken the import of the work of Guyer and Smith (1920), the position is changed. If environment can alter the germ plasm, what environmental factor is in closer relationship with the germ plasm than the soma ? And again, is not the soma brought into still more intimate connection with the germ plasm by the blood stream ? And, further, how otherwise does our treatment reach the germinal area ?

If the value of our experimental results is denied, only one reason for such a course can be advanced—that the strains with which we commenced our work carried melanism in the recessive condition. However, such a view raises more difficulties than it explains. Indeed, we do not admit that it removes any, for the relation of cause and effect in our work seems so perfect that none are left to remove.

If appeal is made to latent melanism, then we are entitled to ask for explanations of the following facts :—

(1) In spite of the fact that *Selenia bilunaria* is a very common species, and very frequently bred, no melanic forms, such as we induced, have ever been encountered in Nature.

(2) We induced the melanism in that species in two different strains, by two distinct methods and in different years.

(3) Similarly, in the case of *Tephrosia histortata*, although a melanic form has been reported from Wales and from the Continent, none is on record for any English county.

(4) In the case of this species also the condition was induced in two distinct series.

(5) In the case of the third species, *Tephrosia crepuscularia*, the melanism was dominant; this cannot possibly be regarded as the emergence of a recessive character.

(6) It is incredible that we, who devised experiments with a certain end in view, should, on four separate occasions, with two distinct species brought from different districts, by the workings of chance, be the only individuals to come into possession of strains carrying latent melanism. This point in itself, in our opinion, affords a perfect answer to any attempts to explain away our results by the "latent recessive" method.

(7) In none of the species employed did the controls ever produce melanic individuals.

(8) When the melanism appeared in our cultures the ratio of the typical individuals to the melanics diverged too widely from Mendelian expectation to permit of the suggestion that we were witnessing the emergence of a recessive character.

(9) Lastly, we are able, almost perfectly, to distinguish the heterozygous type of *S. bilunaria* from the homozygous type; in none of the batches of that species reared prior to the induction of melanism can any insects approaching, even remotely, such heterozygotes be detected.

These facts, to us, seem unanswerable, and we, therefore, see no occasion for pursuing the argument. Reference to one additional point, however, must be made, and that is the possibility that instead of the metal in the compound employed being the active agent, the acid radical may play the part. Further experiments are contemplated at an early date to throw light on the point.

We beg to acknowledge our indebtedness to Mr. J. R. Johnson, and the late Mr. Chas. Robson, who have attended to our cultures in cases of unavoidable absence; and especially to Mrs. Garrett and Mrs. Harrison, who have helped us to rear the larvæ, and without whose help it would have been impossible to experiment on so large a scale.

LITERATURE REFERENCES.

- DONCASTER, L. (1906), "Collective Inquiry as to Progressive Melanism in Lepidoptera," 'Ent. Rec.,' vol. 18.
- GUYER and SMITH (1920), "Further Induction of Eye Defects by Anti-bodies and their Hereditary Transmission," 'Journ. Exp. Zool.,' vol. 31.
- HARRISON, J. W. H. (1920), "Genetical Studies in the Moths of the Geometrid Genus *Oporabia* (*Oporinia*) with a Special Consideration of Melanism in the Lepidoptera," 'Journ. of Genet.,' vol. 9.
- HASEBROEK, K. (1925), "Die prinzipielle Loesung des Problems des Grosstadt- und Industriemelanismus der Schmetterlinge," 'Intern. Ent. Zeit.,' 19 Jahrgang.
- KAMMERER, P. (1906-1913), Various papers in the 'Arch. f. Entwmech.'
- MORGAN, BRIDGES, STURTEVANT (1925). "The Genetics of *Drosophila*," 'Bibliographia Genetica.'
- ULE, C. H. (1925), "Ueber Auftreten und Verbreitung von *Amphidasis betularia* L. f. *carbonaria* Jord. auf dem Kontinent," 'Intern. Ent. Zeit.,' 18 Jahrgang.

*The Diffraction Method of Measuring the Diameters
of Erythrocytes.*

By W. G. MILLAR, M.B., Ch.B., F.R.M.S.

(Communicated by Sir E. Sharpey Schafer, F.R.S.—Received September 30, 1925.)

(From the Department of Physiology, Edinburgh University.)

Pijper (1, 2) has described a method of measuring the diameters of erythrocytes and similar circular bodies, by means of the diffraction spectra produced by them, under suitable conditions.

The principle of the method is simple, and may be summed up as follows. A parallel beam of light is allowed to fall normally on the cell suspension and is then received, together with the light diffracted by the cells, by a lens. This lens converges the light and forms an image of the original light source at its principal focus. The diffracted light is also brought to a focus in the same plane as a series of circular spectra concentric with the central image of the source. The diameter of any given spectrum corresponding to a particular wave-length can be measured, and from this the mean diameter of all the cells producing the diffraction can be calculated.

Pijper derives a simple expression connecting the various factors, from elementary principles, the validity of which will be dealt with later.

If

- f = equivalent focal length of the lens
- r = radius of a spectrum
- n = number of this spectrum from centre
- λ = wave-length of part of spectrum measured
- $2a$ = diameter of cell

$$2a = \frac{n\lambda \sqrt{(f^2 + r^2)}}{r}.$$

The symbols have been altered slightly, the better to conform to the notation to be employed hereafter. This formula may further be modified to a form similar to that usually met with in optics by introducing the diffraction angle θ .

If for the term

$$\frac{r}{\sqrt{(f^2 + r^2)}}$$

we substitute $\sin \theta$ and re-arrange, it becomes

$$2a \cdot \sin \theta = n\lambda. \tag{1}$$

Pijper derives this expression by treating the cell as though it were the opaque portion of a grating element, and entirely neglects the parts of the suspension between the cells, on the grounds that the effects from these cancel out. It is evident that a collection of discs of different sizes lying in a haphazard manner over the field cannot be treated in the simple manner suitable to a regular grating, although Pijper is correct in his assumption that the influence of the spaces cancels.

I am indebted to Prof. C. G. Darwin for the following analysis. The cell will be regarded as a circular disc of negligible thickness, which receives parallel light incident normally. Let E (fig. 1) represent in perspective a circular disc observed in a direction θ to the normal.

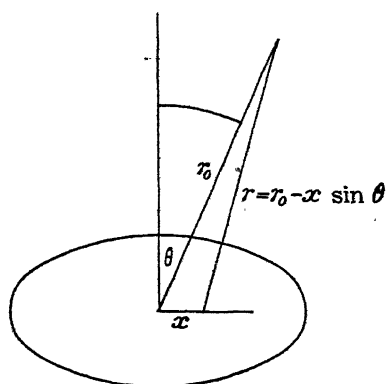


FIG. 1.

From the element of surface ds , defined by co-ordinates x, y , comes a wave proportional to

$$\iint \exp. -ik \sqrt{[(r \sin \theta - x)^2 + (y^2 + r^2 \cos^2 \theta)]} dx \cdot dy,$$

where

$$k = 2\pi/\lambda.$$

Putting

$$x = \rho \cos \phi, \quad a = \text{radius of disc}$$

$$= \int_0^a \int_0^{2\pi} d\phi \cdot e^{-ik(r-x \sin \theta)} \rho d\rho \text{ nearly,}$$

$$= 2\pi e^{-ikr} \int_0^a J_0(k\rho \sin \theta) \rho \cdot d\rho.$$

Putting

$$\begin{aligned}
 z &= ka \cdot \sin \theta \\
 &= 2\pi e^{-ikr} \frac{1}{k^2 \sin^2 \theta} \int_0^{ka \sin \theta} z \left(1 - \frac{z^2}{2^2} + \frac{z^4}{2^2 \cdot 4^2} - \dots \right) dz \\
 &= 2\pi e^{-ikr} \frac{ka \cdot \sin \theta}{k^2 \sin^2 \theta} \cdot J_1(ka \cdot \sin \theta) \\
 &= 2\pi a^2 e^{-ikr} \cdot \frac{J_1(ka \cdot \sin \theta)}{ka \cdot \sin \theta}.
 \end{aligned}$$

This vanishes when

$$J_1(ka \cdot \sin \theta) = 0.$$

This latter is therefore the condition for minimum illumination in the direction θ .

The illumination in this direction will be greatest when

$$\frac{J_1(ka \sin \theta)}{ka \sin \theta}$$

is a maximum, that is, when

$$J_2(ka \cdot \sin \theta) = 0.$$

Solving for values of $ka \cdot \sin \theta$ or z to satisfy the last equation, and rearranging, we have

$$2a = z/\pi \cdot \lambda \cdot \operatorname{cosec} \theta. \quad (2)$$

To prove that with a haphazard arrangement the total effect is the sum of all the small effects, and that the distances between the cells do not enter into the final result, let there be N centres in 1 cm.^2 say, the first at x_1, y_1 , etc.

Taking one at r_0 distance from the centre, we have for this one as its factor

$$\exp. - ik(r_0 - x_1 \sin \theta) = e^{ikx_1 \sin \theta}.$$

The whole lot will give amplitude

$$\sum_{r=1}^N (e^{ikx_r \sin \theta})$$

and intensity

$$\left(\sum_{r=1}^N e^{iqx_r} \right) \left(\sum_{s=1}^N e^{-ikx_s \sin \theta} \right),$$

where

$$\begin{aligned}
 q &= ak \sin \theta \\
 &= N + \sum_r \sum_s 2 \cos q(x_r - x_s)/a \\
 &= N + N(N-1) \cos q(x_1 - x_2)/a.
 \end{aligned}$$

If we average the last expression it is easily seen to vanish. For, take x_1 ,

fixed and let x_2 be anywhere (fig. 2). Its chance of being in a range dx at distance x is dx/l where l is the breadth. Therefore average is

$$\int_{-1/2}^{1/2} \cos q \frac{x}{a} \frac{dx}{l} = 2 \frac{a}{ql} \sin q \frac{l}{2a}.$$

This vanishes on the average, since the sign is as much positive as negative, and to take in the edge values would amount to counting up the diffraction pattern of the edge of the field, obviously not wanted.

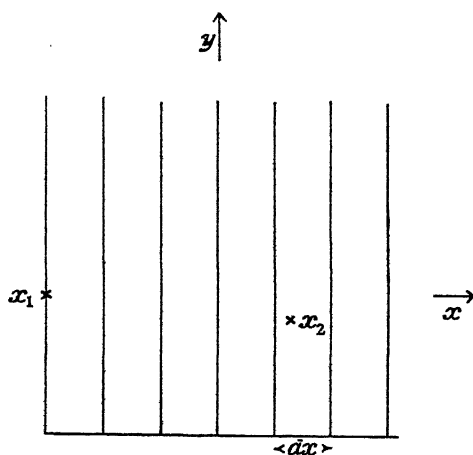


FIG. 2.

The values of z/π for formula (2) may be derived from tables of Bessel functions. Those corresponding to the first five spectra and the intervening dark spaces are given in the Table below.

Table I.

Order of Spectrum.	z/π .	
	Maxima.	Minima.
1	1.63	1.22
2	2.68	2.23
3	3.70	3.24
4	4.71	4.24
5	5.72	5.24

Bergansius (3) quotes a formula very similar to equation (2) but points out that this invariably gives too high values for the diameter. In consequence of this

discrepancy he holds that diffraction by the cells acting as opaque bodies is not the most important cause of the spectra. He claims that the spectra are due to interference from the images of the light source produced by the erythrocytes which act as little lenses. In these circumstances Pijper's formula would hold and would give the mean distance separating the cells, which, in the special case when the cells are in contact, would be equivalent to the mean diameter.

It is obvious that Bergansius's results, if justified, offer a fundamental objection to the diffraction method, the usefulness of which would then be limited by the accuracy with which films could be prepared wherein the cells would just touch, with neither serious overlapping nor wide intervening spaces. It is accordingly necessary to consider this question further, and it will be taken up again in the discussion of results below.

Both Pijper and Bergansius project the spectra on a screen which in the case of the former is opaque. The latter substitutes a ground glass, while both observers work from the linear dimensions of the spectra. This method presents several disadvantages. In the first place linear values for the spectra involve the introduction into the formula of the focal length of the second lens, which is an extra, if not serious, source of inaccuracy, unless its value is carefully determined, since the nominal figure given by the makers is subject to a certain amount of variation. A more important point is the comparatively low intensity of the spectra, which renders it rather difficult to see them on any form of screen. Accordingly in the instrument about to be described the spectra are observed in a pivoted telescope, by which means the value of the diffraction angle θ can be determined directly and also the faint spectra well seen, owing to the smaller loss of light.

In outline the plan adopted is as follows: The image of the light source A is focussed on the pinhole I (fig. 3), which itself is in the principal focus of the

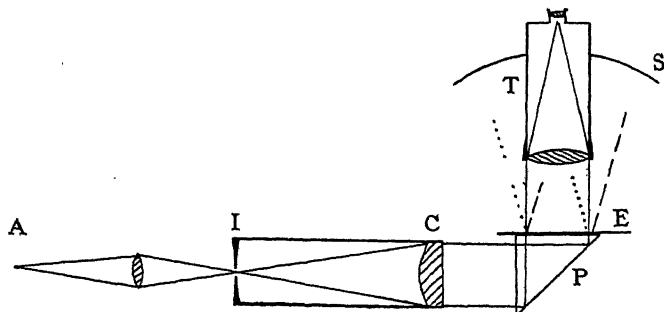


FIG. 3.

collimating lens C and which takes the place of the slit of a spectroscope. The horizontal parallel beam issuing from the collimator is thrown on a right-angled glass prism P and is reflected vertically upwards through the cell suspension E. Here the light is diffracted and passes upward as a central main white beam with a series of spectra arranged concentrically outside it. The diffraction angle is measured by moving the telescope T until the desired diffraction beam passes along its axis and reading off the angle from the vertical on the scale S. The axis of rotation of the telescope is arranged to lie in the plane of the cell suspension.

A general view of the actual instrument is shown in fig. 4 and the construction of the essential parts is as follows.

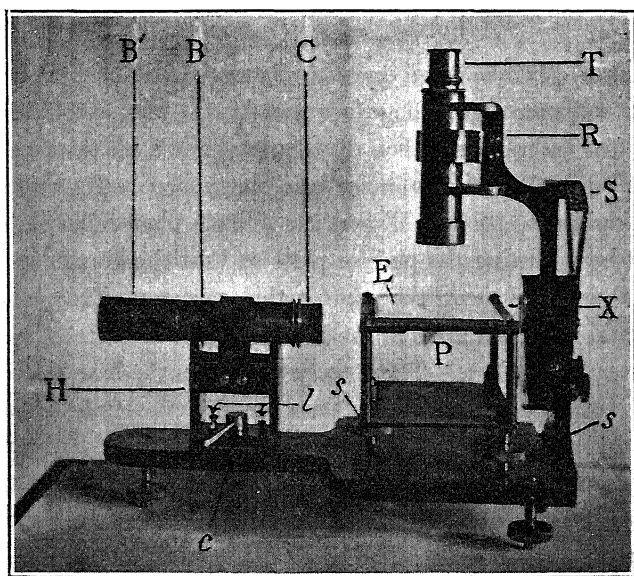


FIG. 4.

The collimating system consists of an F/6 anastigmat photographic lens C of about 6 inch focal length, screwed into a brass tube B, which is rather less than 6 inches long. On the other end of the tube B is another tube B', sliding on it and carrying a flange and cap which hold the pinhole plate. This is simply a circle of ferrotype plate or stencil brass, in the centre of which is a small hole about 0.5 mm. in diameter, formed at the intersection of crosslines marked on the plate. The collimator is attached to the base plate of the instrument in a U-shaped holder H, provided with V-grooves in which the tube B is held by

a spring. The collimator holder has a limited range of adjustments by means of levelling screws *U* and a clamping pivot *c*.

The holder for the reflecting prism and films consists of a plate-glass stage *E* held in a framework which can be levelled by three screws *sss*. To the centre of the lower surface of the stage *E*, a right-angled prism *P* is cemented with Canada balsam.

The telescope *T* is held in V-grooves on an arm *R* which is pivoted about a stout hollow axis *X* attached to a vertical prolongation of the base plate, which also carries a scale of degrees *S* with central zero. The telescope eyepiece is provided with an opaque half diaphragm instead of cross wires, for the purpose of cutting off glare from the central image.

Adjustment of the Instrument.

(i) *Collimation*.—A circle of ground glass is placed on the end of the sliding tube *B'* of the collimator, ground surface towards the lens, and held in position by the cap. The collimator is then directed towards a cloud or other distant object and *B'* adjusted until the image is in sharp focus. The cap is removed without shifting the position of *B'* and the pinhole plate replaced. Centering is carried out by adjusting the pinhole plate so that the crosslines are in continuation of those marked on the cap for this purpose. The pinhole is now accurately at the principal focus of the lens *C*.

(ii) *Adjustment of the Stage*.—The stage is placed on the base plate so that the points of its levelling screws bed into three small conical holes which serve to indicate the correct position. A slide such as will be used for the preparation of the films is laid on the top of the stage, which is then observed through the hollow pivot of the telescope. At the time of manufacture the centre point for the hole in the axis is indicated by cross lines, and these are retained in the finished instrument, one being horizontal the other vertical. The two levelling screws of the stage nearest to the pivot are then adjusted so that the rear edge of the slide is level and collinear with the horizontal crossline. When this has been done, the third levelling screw in front of the stage is adjusted until the line of sight through the axis of the pivot just grazes the top surface of the slide. This adjustment ensures that the axis of rotation of the telescope lies in the plane of the cell suspension, so that the scale readings correspond to the diffraction angles.

(iii) *The Telescope*.—This is adjusted in the usual manner by focussing on a distant object. Owing to the method of adjusting the collimator, the image of the pinhole can be used for this purpose.

(iv) *Adjustment of Alignment.*—To simplify the design of the instrument the optic axis of the telescope is taken to be accurately at right angles to its axis of rotation, and zero error is taken to be absent. Hence the alignment of the collimator has to be adjusted to that of the telescope. The latter is set at zero and clamped. The image of not too bright a light source is focussed on the pinhole and the image of the latter found in the telescope. If it is not in the centre of the field it is made so by adjusting the collimator holder by means of its levelling screws and pivot. When this is done the collimator adjustments are clamped, care being taken that this does not shift the holder.

(v) *Levelling of Stage Surface.*—Before use a spirit-level is laid on the top surface of the stage and the bubble centred by altering the levelling screws of the base plate. Those of the stage must on no account be interfered with or the alignment will be upset.

(vi) *Illumination.*—The instrument having been adjusted it only remains to arrange the light source. A 4-ampere carbon arc is used, and is placed in line with the collimator tube, the positive carbon lying in the optic axis of the collimator. A condensing lens, such as a Nelson bull's-eye, is then arranged near to the pinhole, so that a small image of the positive crater is formed at the intersection of the cross lines on the pinhole plate. When this is done an illuminated circle is seen on the stage, and by altering the iris of the collimating lens C the area of this circle, and hence the area of cells, to produce the diffraction can be adjusted as required. It will be found on applying the eye to the telescope that the central image of the pinhole is exceedingly bright, hence the precaution should always be taken of swinging the telescope out of the centre so that the pinhole image is hidden behind the opaque half diaphragm, or of inserting heavily stained colour screens temporarily.

Once the instrument is set up, it is only necessary to start the arc before use.

Preparation of Films.

In order to use the instrument with any degree of accuracy, particular attention must be devoted to the preparation of suitable films, for it is obvious that two or more overlapping cells will have much the same diffracting effect as a very large cell, so that the presence of rouleaux or partial rouleaux completely vitiates readings. Hence, up to the present, it has not been possible to obtain reliable measurements of cells in pure plasma or serum. On the other hand, cells in saline media are very liable to distortion and crenation, and, moreover, in some cases assume a spherical form (Gough (4)). All these changes are accompanied by alterations in diameter.

The most satisfactory medium so far found is isotonic sodium citrate (1.5 per cent.), to which is added from 10 to 20 per cent. of serum or plasma, derived, of course, from the same source as the cells. It has been found that cells in this medium preserve their shape and size remarkably well, the serum preventing the assumption of the "Goughian" form.

The slides used are made by cutting old quarter-plates in half, and hence measure $3\frac{1}{4}$ in. by $2\frac{1}{8}$ in. Large, fairly thick cover-slips are used, their size being about $2\frac{1}{2}$ in. by $1\frac{1}{2}$ in.

The thickness of film which gives the best results has to be learnt by experiment, and is easily found with a little practice. A given separation of cells can be obtained by a thin film of a rich suspension or by a thick film of a relatively scanty suspension. In general, the latter is the better way, since the cells spread more evenly in the greater thickness of fluid. With the slides described about 0.1 c.c. of a suspension containing 5 per cent. of whole blood (or its equivalent) gives good results.

Owing to the gross errors liable to be introduced by the presence of rouleaux or crenation, it is absolutely essential that all films be examined microscopically before any reliance is placed on readings found with them. The importance of this point cannot be over-emphasized, and measurements made without this precaution are utterly worthless.

Measurement of the Diffraction Angle.

In principle, the taking of readings of the diffraction angle is simplicity itself. All that has to be done is to swing the telescope so that the part of a given spectrum corresponding to the desired wave-length is in the centre of the field and take the scale reading. In practice, however, several difficulties arise.

In the first place, owing to the range of sizes of the diffracting bodies the spectra are not pure and the colours merge into one another to a considerable extent, while the violet ends of the higher-order spectra overlap into the red ends of the lower ones. It is in consequence a matter of no little difficulty to form a satisfactory judgment of the correct position for the telescope. Moreover, even were the spectra pure, the estimation of the wave-length corresponding to a given colour is neither easy nor reliable, and Pijper quite rightly chooses the yellow portion, which is the most narrowly confined colour.

It is clear, therefore, that improvement is very necessary in this direction. The immediately obvious step is to use monochromatic light. Unfortunately

a very intense light source is needed, for even with the carbon arc the higher spectra are not sufficiently bright to read with any degree of comfort. Hence the ordinary sodium flame and the mercury-vapour lamp are useless. The sodium-cored carbons for the ordinary arc are of little more use, owing to the comparatively low light intensity of the arc itself and the enormous preponderance of white light from the craters.

So far no satisfactory source of approximately monochromatic light has been found, and recourse must be had to light filters. Here again success is only partial, for all filters of sufficiently sharp "cut" stop a great deal of light even in the band transmitted, so that the spectra are very faint. Still filters that pass a fairly broad band can be used, and are a distinct improvement. The one found most useful is Zettnow's copper nitrate—chromic acid solution in a thickness of about 1 cm. This passes a pretty sharply cut band in the yellow green, the lower limit of which is clear and lies at $\lambda = 6400$ A.U.

Results and Discussion.

The remainder of the paper will be devoted to the consideration of certain experiments designed to determine what reliance can be placed on measurements of erythrocytes by the diffraction method, comparison being made with figures derived by photo-micrography, for the technique of which reference should be made to a paper by Ponder and the writer (5). In order to aid fluency, discussion of the results will be combined with the enumeration.

In all cases measurements have been made in a medium of 1.5 per cent. sodium citrate plus 20 per cent. plasma, which in the tonicity experiment is diluted as required with distilled water. The Zettnow filter is used, and the end of the red ($\lambda = 0.64 \mu$) of the second spectrum taken. The value of z/π for the second spectrum is 2.68, which, multiplied by 0.64, gives 1.7, by which constant the cosecant of the diffraction angle θ is multiplied to give the diameter. Human cells from the same individual are used throughout and the essential microscopic precautions taken.

I. The Mean Size of Human Erythrocytes.

For this experiment blood is drawn from the finger and 0.1 c.c. added to 2 c.c. of the citrate-plasma solution. The percentage of plasma is thus a little over 20, but there is no tendency to rouleaux formation. After well mixing, about 0.1 c.c. is placed in the centre of a thoroughly clean slide and the cover quickly applied to ensure even distribution. About eight readings can be

taken on each slide, which, containing the equivalent of 0.005 c.c. of whole blood, represents about 25,000,000 cells. The mean of the readings obtained by the diffraction method are given in Table II below.

Table II.

θ .	$\operatorname{cosec} \theta$.	d .
$9^{\circ} 36'$	5.996	10.2μ

It will be seen from these figures that Bergansius's objection that the diffraction formula 2 gives results too high by 10-20 per cent. is confirmed, the mean value by the photographic method being 8.8μ . As mentioned above, Bergansius has discarded the pure diffraction theory in favour of one based on the formation of images of the light source by the cells, which he holds act as little lenses, and claims that it is interference of the light from these images which is the main cause of the spectra. If this be the case, then the diffraction angle or width of the spectra should be much less when a thin film with well-scattered cells is used than when the cells are close together. Bergansius finds this to be the case. The following experiment is described to investigate this point.

II. *Effect of Separation of the Cells.*

Films are made precisely as described in the previous experiment, with the exception that one end of the cover-slip is supported by a small piece of another cover to form a wedge-shaped film. Flat films containing different proportions of cells and citrate-plasma medium have also been measured and give similar results. Readings are taken of parts so thin that the spectra are only just visible and also of parts so thick that the scattering of light is nearly great enough to prevent the spectra being measured. The figures are given in Table III below under the respective headings of "Thin" and "Thick."

Table III.

Film.	θ .	$\operatorname{cosec} \theta$.	d .
Thick.....	$9^{\circ} 36'$	5.996	10.2μ
Thin	$9^{\circ} 15'$	6.222	10.6

It will be seen that there is very little difference in the diameters obtained in the two cases, and certainly no relation between these and the mean separation of the cells, for in the thin films the cells averaged from 2—10 cell diameters apart, while in the thick films they overlapped to some extent. These results must therefore be taken to unequivocally negative Bergansius's theory of lens action and interference.

The cause of the excessive diameter given by the diffraction method is not very easily seen. It must be remembered, however, that the formula used is derived on the assumption that the cells act as though they were flat discs, and no account is taken of their finite thickness nor of the curvature of their edges. Hence there seems no good reason to discard the diffraction explanation in these circumstances.

The conclusion that may justifiably be drawn is that at present the diffraction method is not a reliable one for obtaining absolute measurements of erythrocytes. In view of the considerations as to the influence of the shape of the edge of the cell on the size of the spectra, one is entitled to retain some doubt as to the quantitative reliability of the method for relative measurements. In order to investigate the claims of the method in this respect, certain size changes of erythrocytes have been followed under the "diffractometer."

III. *Effect of Hypotonicity on the Diameter.*

Blood is drawn from the median basilic vein, a small amount of potassium oxalate (10 mgms. per 10 c.c.) being added to prevent clotting. This has been found (5) to have no appreciable effect on the diameter of the cells. Part of the blood is centrifuged to obtain the plasma, the remainder being used for the cells. A series of dilutions of the citrate-plasma mixture is made in various tonicities and the cells added in the proportion of 0.1 c.c. to 2 c.c. of the medium. Films are prepared and at once measured, a control being at the same time examined under the microscope. The results are given in Table IV.

Table IV.

Tonicity Plasma = 1.	θ .	cosec θ .	d .
1 — 0.61	9 36	5.996	10.2 μ
0.50	9 45	5.905	10.0
0.44	9 56	5.797	9.8
0.33	10 30	5.487	9.3
0.25	10 45	5.356	9.1

A progressive decrease in diameter is shown as the tonicity falls. This is in full accordance with expectation if the erythrocyte is regarded as a balloon, and the results agree in kind with those found by Ponder and Millar (6) using the photo-micrographic method. Moreover, the degree of the decrease in diameter is of the same order but rather smaller. If the explanation of the large values found by the diffraction method is to be sought in the curvature of the cell edge, then this smaller degree of decrease is easily intelligible. For, as the major axis of the cell shrinks, the minor axis elongates and the cell departs still more from the form of a flat disc of negligible thickness, so that errors resulting from the original assumption would be increased. The nett observed decrease in diameter would thus be smaller than the actual decrease.

IV. *Alteration in the Diameter during Saponin Hæmolysis.*

To a suspension of cells in the citrate-plasma mixture is added saponin to the concentration of 1/30,000. The high concentration is necessary owing to the inhibitory action of the plasma and to the low temperature (15° C.) which would otherwise necessitate long periods before the action was manifest. Films are made at once and measured at intervals. The results are contained in Table V.

Table V.

Time.	θ .	$\operatorname{cosec} \theta$.	d .
0	9 36	5.996	10.2 μ
2 mins.	12 18	4.694	8.0

No effect is observed for some time, and there is then a rapid decrease in diameter. Almost immediately after this decrease the spectra fade away owing to hæmolysis, and no further observations can be made. The result of this experiment is further evidence against the validity of Bergansius's lens theory, for it is inconceivable that the cells in a film should remain at a fixed average separation, and then after two minutes' action of the saponin suddenly approximate. The figures given above are quite in accordance with those found by Ponder (7) using photography, the same qualifications applying in regard to the quantitative aspect, as were noted in the previous experiment.

V.—Effect of Drying on the Diameter of Erythrocytes.

It is well known that the erythrocyte shrinks in diameter on drying, and a good many experiments have been performed with a view to determining how the diffraction method would show the change. Very much contrary to expectation, no significant difference has been found between the diameters of cells in the citrate-plasma medium and the same cells dried on the slide. Nor has any difference been noted in any medium, even when the dried cells are afterwards fixed in methyl alcohol. This fact by itself lends strong support to Bergansius's theory.

If the matter is considered further, it must be granted that the condition of a cell in the fresh state and that of the same cell dried on the slide cannot be regarded as comparable. In view of the evidence against Bergansius's views it would seem that the explanation is to be sought elsewhere. Admittedly, it is not easy to formulate an entirely satisfactory explanation of the result, but the following suggestion, offered tentatively, may have some bearing.

As the film dries and the liquid decreases it will tend to gather round the edge of the cell, and, when drying is complete, most of the solid matter will be deposited in this position. In direct methods of measurement this circumstance will not affect the results, since each reading is taken to the actual edge of the cell. On the other hand, the aggregation of solid matter may effect the diffraction of the light, so that too large a value is given by the diffraction method.

Conclusions.

As a result of the foregoing, the following conclusions appear to be justified :—

In the first place, the diffraction method is not a reliable means of determining the absolute sizes of erythrocytes.

Secondly, changes in the size of erythrocytes can be followed during the course of an experiment with considerable delicacy, alterations of the order of 0.3μ corresponding to a difference in angle of about $15'$, which is quite readable. On the other hand, the measurement of these differences is to some extent uncertain, owing to the formula probably requiring modification for change of shape of the cell. The instrument is thus somewhat analogous to the gold-leaf electroscope.

The utility of the method is considerably restricted, owing to the disturbing effect of crenation and rouleaux formation, to avoid which special media are necessary and microscopic control essential.

Further improvement seems possible in the provision of sources of mono-

chromatic light of great intensity, and in the derivation of the fundamental formula from assumptions that agree better with the known shape of the cell. The analysis of diffraction caused by bodies of the shape of the red cell, and its extension to cover the alterations in shape, would seem, however, a matter of very great difficulty.

This research was carried out during the tenure of a Carnegie Research Scholarship, the expenses being defrayed by a grant from the Royal Society.

Summary.

1. The theory of the diffraction micrometer is discussed and a formula deduced for a haphazard distribution of cells.
2. An improved form of instrument is described in which certain disadvantages are eliminated.
3. The method is applied to a variety of size changes of human erythrocytes to determine its reliability.
4. The results show that the method is not reliable for the determination of absolute sizes, and although small changes of size can readily be observed, the measurement of these changes is also to some extent unreliable.

BIBLIOGRAPHY.

- (1) Pijper, A., 'Med. Journ. South Africa' (August, 1918).
 - (2) Pijper, A., 'Med. Journ. South Africa' (June-July, 1919).
 - (3) Bergansius, F. L., 'Pfüger's Archiv,' vol. 112, p. 118 (1921).
 - (4) Gough, A., 'Biochem. Journ.,' vol. 18, p. 202 (1924).
 - (5) Ponder, E., and Millar, W. G., 'Quart. Journ. Exper. Physiol.,' vol. 14, p. 67 (1924).
 - (6) Ponder, E., and Millar, W. G., 'Quart. Journ. Exper. Physiol.,' vol. 15, p. 1 (1925).
 - (7) Ponder, E., 'Quart. Journ. Exper. Physiol.,' vol. 14, p. 333 (1924).
-

*The Metabolism of the Diabetic Individual during and after
Muscular Exercise.*

By K. S. HETZEL and C. N. H. LONG.*

(Communicated by Prof. A. V. Hill, F.R.S.—Received October 20, 1925.)

(From the Medical Unit, University College Hospital, and the Department of Physiology
and Biochemistry, University College, London.)

CONTENTS.

	Page.
Introduction and Methods	279
Part. I—(A) The oxygen requirement and the oxygen debt of exercise.....	281
(B) Lactic acid formation and removal	284
Part II.—The respiratory quotient of the excess metabolism.....	286
Part III.—Observations on the blood sugar	292
Part IV.—(A) The formation and oxidation of ketones.....	299
(B) The urinary nitrogen	302
(C) The sugar excretion in the urine	302
Summary	303
Appendix and References.....	305

Introduction.

The work of Hill, Long and Lupton (1) on the effects of muscular exercise in normal men, and more recently that of Furusawa (2) on the carbohydrate metabolism during exercise, made it desirable to undertake a similar investigation of the reactions to exercise of a diabetic individual with his deficient power of utilizing carbohydrates. Furusawa's results show that in normal men on an ordinary diet the respiratory quotient of the excess metabolism due to exercise is unity, for periods of moderate exercise up to about 20 minutes. For similar exercise of longer duration the respiratory quotient of the excess metabolism is lower. When the subject had been living for some time previous to the experiment on a diet consisting chiefly of fat, the respiratory quotient of the excess metabolism was still unity for exercise of very short duration, but fell below unity for efforts of much shorter duration than 20 minutes. In the diabetic the carbohydrate reserves are less than in a healthy man, and his diet is restricted as regards carbohydrate and contains an excess of fat to make up the requisite number of calories. The diabetic, therefore, under such

* Working for the Industrial Fatigue Board of the Medical Research Council.

conditions, might be expected to approximate, in regard to exercise, to a healthy man living on a diet largely composed of fat. In the present investigation the effect of muscular exercise has been studied in three diabetic patients on lines similar to those adopted by the above authors. We hoped that by exercising these patients with and without injections of insulin, some conception might be formed of the rôle of insulin in the metabolic processes involved in muscle during and after exercise.

Recent work by Himwich, Loebel and Barr (3), and by Doisey, Briggs, Weber and Koechig (4), has shown that in the diabetic animal the formation of lactic acid from the glycogen of muscle on exercise is independent of the presence of insulin. Our results on diabetic patients confirm this view. We desired also to ascertain the likelihood of the development of ketosis and diabetic coma as the result of exercise in diabetics, together with the modifications which insulin might have on this process; further, there was the question of restriction of exercise in such patients, if it acted deleteriously. We attempted, moreover, to obtain information as to whether the dosage of insulin required depended on the amount of muscular work which the individual undertook, and also as to the need for regulation of exercise after a patient had been discharged from the inactive life in hospital.

Methods.—The three male patients whom we have employed for these investigations had been treated for diabetes mellitus, by adjustment of diet and injections of insulin for some time previous to the beginning of our experiment, a time varying from one month to twelve months. It had been attempted to render them aglycosuric and with normal blood-sugar fluctuations during this period. The diet and dosage of insulin were kept constant throughout, except that in one or two experiments the insulin was increased temporarily. The observations extended over twelve months. The extent of the diabetes was of moderate severity in two subjects (J. Th., aged 16, and E. S., aged 36) and severe in one (F. T., aged 21). Further clinical details are given in the Appendix.

The observations fall into three groups, depending upon the time of the last injection of insulin, namely: (1) insulin injected within the last six hours, (2) without insulin for periods from 10 to 17 hours, (3) without insulin for 20 to 27 hours. Meals were kept constant in amount and as regards the time of partaking. Where results are compared the observations were made at the same time of day in the case of each subject, except only in a few experiments, where for various reasons the time was altered.

The exercise in all experiments was that of "standing running" for various

times and at different rates. The rate chiefly employed was 156 steps per minute, timed to a metronome. This rate is only moderate exercise for a healthy man on a normal diet. A fore-period was allowed, varying from 20 to 60 minutes, during which the subject rested before any respiratory observations were taken. A resting sample of expired air was collected over a period from 10 to 15 minutes long, to allow a determination of the initial resting metabolism; then from the beginning of exercise till the end of recovery the expired air was collected in Douglas bags of various sizes. At the end of the recovery period another resting sample of expired air was collected for 10 to 15 minutes, and if the initial and final resting metabolisms did not agree within reasonable limits the experiment was discarded.

The urine was collected before the experiment began and at varying intervals during the recovery period. Estimations of glucose were made by Benedict's method and of total nitrogen by the Kjeldahl method on these specimens. The urinary ketones were examined by a rough quantitative method of diluting the urines to equal volumes, and observing the colour produced in one minute by means of the Rothera sodium-nitroprusside test. All the specimens from one experiment were examined at the same time and the tubes set side by side so that the colours might be compared simultaneously. The depth of colour was recorded by a symbol varying from — to +++.

Blood samples for sugar estimation were taken several times during an experiment, at least once every half-hour, and often more frequently. The glucose content was estimated by Maclean's (5) method, the small amount of blood required (0.2 c.c.) permitting frequent samples to be taken by pricking the lobule of the ear. For lactic acid estimations by Clausen's method (6) blood was withdrawn from the median basilic vein, as also for the estimation of acetone bodies by Shaffer's (7) and van Slyke's (8) methods. These blood samples were taken before exercise, immediately after exercise, and at the end of recovery. In some cases, where the ketones alone were being estimated, the blood was taken at the beginning and at the end of the experiment.

Part I.—(A) The oxygen requirement and the oxygen debt of exercise.

The first question which we sought to answer was, are the processes involved in muscular exercise the same in the diabetic as they are in the normal man? Hill, Long and Lupton, in their experiments on normal men, studied the oxygen intake, the oxygen debt, the oxygen requirement and the duration of the recovery following exercise of varying severity. They have shown the inter-relations of these several quantities. If the exercise be mild, the oxygen

requirement is readily met by the actual oxygen intake of the body; consequently, the oxygen debt is small and recovery rapid. On the other hand, if the exercise be severe, it may not be possible to meet the oxygen requirement by oxygen intake during the exercise; the oxygen debt becomes large and recovery is protracted. These relations have been studied in our three diabetic subjects. (See Table I and fig. 1.)

Table I.—Recovery Data, Subject F.T.

Experiment 1.—156 steps per minute for 5 minutes. Resting, $\text{CO}_2/\text{O}_2 = 207/308 = 0.68$; ventilation, 5.7 litres per minute; exercise (last minute), $\text{CO}_2/\text{O}_2 = 1560/1820 = 0.85$; oxygen debt (26½ minutes' collection) = 1500 c.c.

Intervals of collection*	15"	30"	1'	5'	10'	10'
Mid point of interval	7½"	30"	1' 15"	4½'	11½'	21½'
O ₂ c.c. per minute	1,780	1,158	825	370	279	315
R.Q.	0.81	0.92	0.92	1.0	0.76	0.73
Ventilation, litres per minute	36.2	26.6	19.8	10.9	6.9	7.0

Experiment 2.—220 steps per minute for 3½ minutes. Resting values as in Experiment 1. Exercise (last minute), $\text{CO}_2/\text{O}_2 = 2250/2530 = 0.89$; oxygen debt (26½ minutes' collection) = 3300 c.c.

Duration of interval	15"	30"	1'	5'	10'	10'
Mid point of interval (from end of exercise)	7½"	30"	1' 15"	4½'	11½'	21½'
O ₂ c.c. per minute	1,830	1,502	846	564	362	306
R.Q.	0.85	0.93	1.04	0.91	0.82	0.71
Ventilation, litres per minute	39.2	31.8	23.0	13.7	8.95	6.86

* Starting from end of exercise.

SUMMARY.

—	Exercise.	O ₂ Requirement of Exercise, c.c. per min.	Max. O ₂ Intake, c.c. per min.	O ₂ Debt, c.c.	Completion of Recovery.
Experiment 1	156 steps per minute for 5 minutes	2,100	1,820	1,500	12 mins.
Experiment 2	220 steps per minute for 3½ minutes.	3,500	2,530	3,300	27 mins

Fig. 1 represents the two curves obtained by plotting the excess oxygen used during recovery from exercise, with the time as abscissa, for mild and more strenuous exertion respectively. If these recovery curves be compared with those of normal individuals who have undertaken similar exercise, they

will be found to be similar both in shape and as regards the time required for the return of the subject to his resting condition.

The oxygen intake and the maximum oxygen debt for this subject (F.T.), as also for the others though to a lesser degree, appear to be small, and the more strenuous exercise caused him fairly severe exhaustion: he appeared not to be capable of the intense efforts which can be made by normal healthy individuals. The oxygen intake in Experiment 2 being obviously somewhere

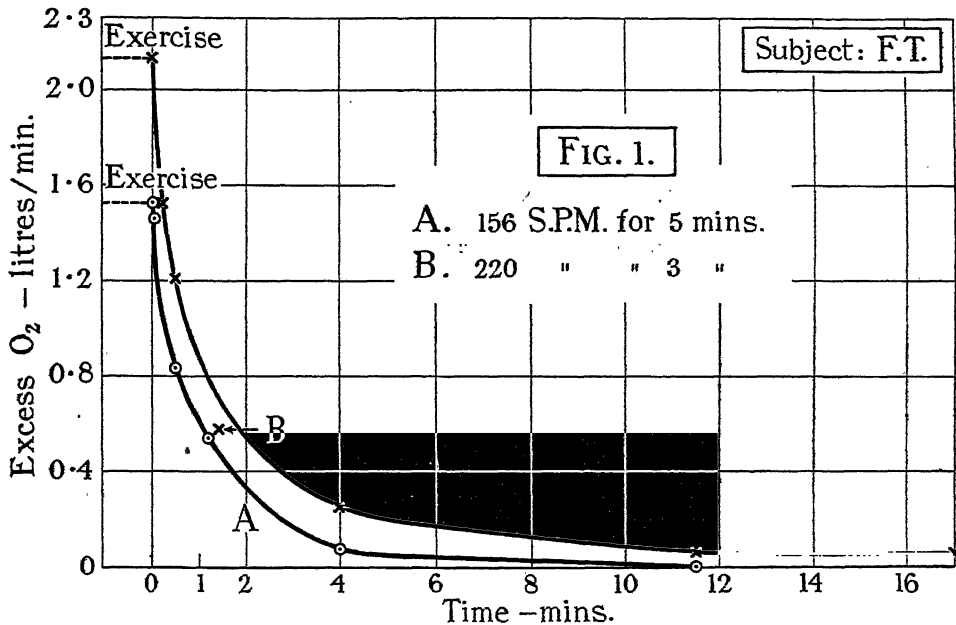


FIG. 1.—Oxygen intake during recovery. Horizontally, time from cessation of exercise: vertically, rate of oxygen intake in litres per minute in excess of resting. Exercise ended at zero time. Subject F.T. Curve A: Exercise of 156 steps per minute for 5 minutes, showing curve rapidly falling to base line. Curve B: Exercise of 220 steps per minute for 3 minutes. Curve, falling less rapidly, showing slower recovery from the more strenuous exercise of less duration.

near its maximum, the oxygen debt could no doubt be exceeded by substituting more violent exercise of shorter duration. In E.S., a less-severe diabetic, oxygen debts of 5 to 6 litres have been noted, while his maximum oxygen intake is about 3 litres per minute.

The respiratory quotient following exercise shows the usual feature of a rise followed by a fall. The rise does not appear to be as pronounced as in the normal, but it must be remembered that the diabetic starts with a much lower resting respiratory quotient. The phenomenon of a carbon-dioxide retention

in the later stages of recovery, with its consequent fall in the respiratory quotient below the resting level, also has been observed, but no detailed study has been made of this point.

(B) *Lactic acid formation and removal.*

Among the numerous syntheses and breakdowns which carbohydrate undergoes in the body is the conversion of glycogen, possibly through some intermediary, into lactic acid, this being followed during recovery by the re-conversion of lactic acid into glycogen. It was of interest, therefore, to determine whether, following muscular exercise in diabetics, the production and removal of lactic acid were similar to those occurring in normal individuals. Table II contains observations of the amount of lactic acid in the venous blood of a subject before, during, and following both mild and severe exertion.

Table II.—Lactic Acid in Blood.

Date of Experiment.	Length and Severity of Exercise.	Subject.	Moment of Observation.	Lactic Acid in Blood. Mgms. per 100 c.c.	Remarks.
8/11/24	156 steps per minute for 8 minutes	J.Th.	Rest	28.0	With insulin.
			Immediately after exercise	78.0	
			103 minutes after exercise	26.6	
25/11/24	Ditto for 8 minutes	J.Th.	Rest	13.8	Without insulin 16 hours.
			Immediately after exercise	21.4	
			99 minutes after exercise	11.7	
4/12/24	Ditto for 8 minutes	J.Th.	Rest	47.0*	Without insulin 29 hours.
			Immediately after exercise	76.5	
			102 minutes after exercise	49.5*	
27/2/25	Ditto for 8 minutes	F.T.	Rest	20.4	Without insulin 23 hours.
			Immediately after exercise	38.2	
			111 minutes after exercise	25.9	
26/11/24	Ditto for 8 minutes	F.T.	Rest	21.4	Without insulin 20 hours.
			Immediately after exercise	52.9	
			112 minutes after exercise	27.5	

* It would seem doubtful whether all of this represents lactic acid.

Table II—(contd.).

Date of Experiment.	Length and Severity of Exercise.	Subject.	Moment of Observation.	Lactic Acid in Blood. Mgms. per 100 c.c.	Remarks.
22/1/25	Ditto for 8 minutes	F.T.	Rest Immediately after exercise 103 minutes after exercise	15.1 26.3 14.7	Without insulin 10 hours.
14/6/24	210 steps per minute for 3½ minutes	F.T.	Rest 86 minutes after exercise	18.6 19.7	With insulin.
17/6/24	Ditto	F.T.	Rest 100 minutes after exercise	36.8 21.0	Without insulin 13 hours.
19/6/24	"All out" 101 seconds.	F.T.	Rest 95 minutes after exercise	21.6 16.2	Without insulin.
12/6/24	"All out" 50 seconds.	F.T.	Rest 70 minutes after exercise	32.4 29.0	With insulin.
19/1/25	156 steps per minute for 8 minutes	F.T.	Rest Immediately after exercise 108 minutes after exercise	21.1 36.5 26.4	With insulin.
15/5/24	210 steps per minute for 3½ minutes	E.S.	Rest 4 minutes after exercise 45 minutes after exercise	28.4 93.4 65.1*	With insulin.
3/6/24	Ditto	E.S.	Rest 2 minutes after exercise 120 minutes after exercise	21.0 62.0 13.7	Without insulin.
22/5/24	Ditto	E.S.	Rest 2 minutes after exercise 110 minutes after exercise	28.9 86.9 30.3	Without insulin 16 hours.
14/12/24	156 steps per minute for 8 minutes	E.S.	Rest 3 minutes after exercise 114 minutes after exercise	23.8 50.9 26.2	Without insulin 13 hours.
15/2/25	Ditto	E.S.	Rest 3 minutes after exercise 83 minutes after exercise	14.1 53.2 22.4	Without insulin 27 hours.

* This higher figure is presumably due to the short period of recovery permitted.

If these results be compared with those given by Hill, Long and Lupton (1) for similar experiments on normal men, it will be seen that the character of the response to muscular exercise, as evidenced by the increase and the subsequent decrease in the lactic acid concentration in the blood, is the same both in diabetics and in normal individuals.

In some of these experiments the increase in lactic acid was not as great as in others, where apparently the same exercise had been taken for a similar time. It is probable that in these experiments the subject did not put so much effort into the exercise and that he flagged towards the end. In the case of the subject E.S., who is a fairly fit man and always did his best at the exercise, the increase in lactic acid is consistently large and quite comparable with that occurring in normal men under similar conditions.

In normal men the general result of previous experiments is that in strenuous exercise the lactic-acid content of the blood rises to values of about 70 mgms. per 100 c.c. In mild exercise it hardly ever reaches such figures. If our observations on diabetic patients be examined from this point of view it will be seen that they are closely analogous to the results obtained on normal men. The time required for the removal of the extra lactic acid produced by exercise is also about the same in both cases: moreover, in the diabetic subject the result appears not to be affected by the presence or absence of exogenous insulin.

It would seem, therefore, that the production of lactic acid from glycogen and the subsequent removal of this lactic acid during recovery are not dependent on the presence or absence of insulin in the body. We are able in this respect to confirm the work of others on this phase of carbohydrate metabolism.

Part II.—The respiratory quotient of the excess metabolism.

By the excess metabolism is implied the total oxygen used and the total carbon dioxide expired as the result of an interval of exercise, over and above the resting values for an equal period of time. The process considered involves both exercise and recovery. The respiratory quotient of this excess metabolism is an index of the metabolic changes, provided that certain precautions are taken. The essential condition is that at the end of the period of collection of the expired gases the subject should have completed his recovery and be in the same bodily condition as when the exercise commenced. Thus the hydrogen ion concentration of his tissue fluids should be at the resting level again. Indications of this are provided by a measurement of his lung ventilation, or of

the partial pressure of carbon dioxide in his alveolar air. Further, the lactic acid content of his blood should have returned to its previous level and his final resting metabolism should be identical with that before exercise; otherwise the experiment from this point of view is of no value. When these conditions are satisfied the excess oxygen usage and the excess carbon dioxide eliminated are due only to the exercise, and the respiratory quotient gives us an idea of the nature of the metabolism associated with the exercise.

The experimental methods employed and the results obtained on normal men have been described in full in the papers of Hill, Long and Lupton (1) and of Furusawa (2). In the present investigation the chief interest lies in the respiratory quotient of the excess metabolism rather than in the actual magnitude of the oxygen requirement for any given type of exercise. The essential feature of Furusawa's results was that normal men, on a normal diet, possessed sufficient stores of carbohydrate, immediately available, to enable them to exercise at a moderate rate (about 150 steps per minute) for an appreciable time, up to 20 minutes, at the expense of carbohydrate alone. As the result of longer intervals of exercise other substances begin to be utilised. If the carbohydrate stores had been previously depleted, as the result of subjection for some days to a diet largely composed of fat, then the utilisation of other substances came into operation earlier,—at 150 steps per minute after a few minutes only. This point has been investigated in some detail in our three subjects. We have performed altogether some fifty experiments on this aspect of the question, the time of exercise varying from half a minute to 8 minutes. Longer periods of exercise were not considered justifiable on account of the condition of the subjects, and were indeed not necessary, as our results show. The time allowed for recovery from the exercise is of the greatest importance. We have given 30 minutes recovery for the half-minute intervals of exercise, and up to 2 hours for the 8-minute periods.

The results obtained are similar in type but different in degree for the three subjects. In every case the respiratory quotient at rest was low, as was to be expected from the diet on which the three subjects were maintained. The experiments can be divided into three categories, (a) in which insulin had been administered during the previous six hours, (b) in which the last dose of insulin had been given 10 to 17 hours previously, and (c) in which the subject had been without insulin for 20 to 30 hours. The following table shows that no serious change was produced in the resting respiratory quotient by the administration or the absence of insulin.

Table III.—Respiratory Quotient of the Excess Metabolism. Exercise, standing running at 156 steps per min. in all Experiments.

A. Subject E. S.

No. of Exp.	Duration of Exercise, Mins.	Mean Resting Value, CO_2/O_2 , c.c. per Min.	Resting R.Q.	Time of Collection, Mins.	Total Metabolism, CO_2/O_2 (c.c.).	Excess Metabolism, CO_2/O_2 (c.c.).	R.Q. of excess metabolism.	Remarks.
1	0.5	274/387	0.72	32	10450/14230	1700/1805	0.92*	Insulin within last 6 hours.
2	1.0	286/373	0.77	41	15154/18732	3394/3432	0.99*	" " "
3	3.0	212/258	0.82	40	16970/17620	8490/7320	1.16*	" " "
4	4.3	295/408	0.74	74	31580/39140	9780/8940	1.09*	" " "
5	5.0	212/269	0.79	65	29630/32540	15850/15060	1.05*	" " "
6	8.0	277/371	0.75	81	35950/44250	13550/14250	0.95*	" " "
7	8.0	212/290	0.73	102	41479/59085	19885/19555	1.02*	Insulin within last 6 hours.
8	3.0	301/381	0.79	68	31220/35072	10970/9222	1.09*	O_2 intake during exercise 2125 c.c. per min. Insulin within last 10-17 hours.
9	8.0	212/284	0.75	85	44900/55270	26900/32020	0.86*	" " "
10	8.0	328/406	0.80	114	65409/73456	28009/26956	1.04*	" " "
11	0.5	305/384	0.79	31	10904/13934	1454/2034	0.72†	Without insulin 20-30 hours.
12	5.0	284/375	0.73	73	28021/38889	7241/11489	0.63†	" " "
13	8.0	266/340	0.78	71½	35808/43085	16808/18285	0.90†	Without insulin 20-30 hours. O_2 intake during exercise 1980 c.c. per min.

B. Subject J. Th.

1	0.5	223/304	0.74	17.0	5300/6830	1510/1670	0.91*	Insulin within last 6 hours.
2	1.0	203/287	0.71	27.0	8960/11100	3480/3350	1.03*	" " "
3	3.0	195/278	0.70	48.0	16960/21965	7600/8645	0.89*	" " "
4	5.0	191/238	0.81	57.0	26140/30810	15260/17230	0.89†	" " "
5	8.0	216/312	0.70	103.0	38567/50497	16347/18347	0.89†	" " "
6	1.0	217/302	0.72	26.0	9500/11630	3600/3780	0.95*	Insulin within last 10-17 hours.
7	3.0	232/311	0.76	53.0	18680/22850	6186/6050	1.02*	" " "
8	5.0	214/289	0.74	73.0	27356/34010	11756/12910	0.91†	Insulin within last 10-17 hours. O_2 intake during exercise 2128 c.c. per min.
9	8.0	241/323	0.75	99.0	35220/46590	11400/14590	0.78†	Insulin within last 10-17 hours.
10	5.0	179/234	0.77	88.0	26084/35611	10364/15011	0.70†	Without insulin 20-30 hours.
11	8.0	240/301	0.80	102.0	39780/48494	15330/17794	0.86†	Without insulin 20-30 hours. O_2 intake during exercise 1874 c.c. per min.

Table III—continued.

C. Subject F. T.

No. of Exp.	Duration of Exercise, Mins.	Mean Resting Value, CO ₂ /O ₂ , c.c. per Min.	Resting R.Q.	Time of Collection, Mins.	Total Metabolism, CO ₂ /O ₂ (c.c.).	Excess Metabolism, CO ₂ /O ₂ (c.c.).	R.Q. of excess metabolism.	Remarks.
1	1.0	123/156	0.79	30	6456/8146	2746/3440	0.80*	Insulin within last 6 hours.
2	3.5	225/290	0.78	86	26880/32190	7530/7300	1.04*	" " "
3	5.0	147/192	0.77	59½	16360/20620	7610/9200	0.83½†	" " "
4	8.0	244/332	0.71	108	41657/52041	15257/10141	0.95½†	" " "
5	8.0	128/173	0.74	75	22830/28695	9600/12960	0.84½†	" " "
6	3.5	193/239	0.81	100	29530/34540	10230/10640	0.96*	Insulin within last 10-17 hours.
7	5.0	259/341	0.76	76½	28960/37050	9160/10550	0.87½†	" " "
8	8.0	238/295	0.81	103	37228/48292	12782/17892	0.72½†	Insulin within last 10-17 hours. O ₂ intake 2340 c.c. per min.
9	0.5	221/296	0.75	31	8468/11148	1618/1898	0.86†	Without insulin 20-30 hours.
10	1.0	218/288	0.77	36	10810/14170	2745/3240	0.85†	" " "
11	3.0	191/262	0.73	58	11070/15190	9995/12155	0.82†	" " "
12	5.0	196/265	0.74	71	23470/30230	9570/11430	0.84†	" " "
13	5.0	193/260	0.74	94	26906/35344	8786/10944	0.81†	" " "
14	8.0	236/323	0.73	112	34412/45780	7962/9580	0.83†	" " "
15	8.0	177/235	0.75	111	32266/42729	12580/16629	0.76†	" " "

* The average value for the R.Q. of the excess metabolism in these 18 cases is 0.99, as compared with an average resting R.Q. of 0.76—see text. (Category x.)

† The average value for the R.Q. for the excess metabolism in these 9 cases is 0.85, as compared with an average resting R.Q. of 0.76. (Category y.)

‡ The average value for the R.Q. of the excess metabolism in these 12 cases is 0.80, as compared with an average resting R.Q. of 0.76. (Category z.)

Table IV.—Respiratory Quotient at Rest: Average of Results in Table III.

Category.	Subject F. T.	J. Th.	E. S.	Average.
a	0.76	0.73	0.76	0.75
b	0.79	0.74	0.78	0.77
c	0.74	0.78	0.77	0.76

Average of all = 0.76

The average respiratory quotient for all the experiments on the resting metabolism is 0.76, which implies that of the energy utilised by the body at rest some 18 per cent. came from the oxidation of carbohydrate and 82 per cent. from the oxidation of fat (neglecting any correction for the amount of protein being metabolised, and counting protein as fat).

The oxygen intake at rest in the three subjects is given in Table V.

Table V.—Oxygen Intake at Rest: c.c. per minute: Average of Results in Table III.

Category.	Subject F. T.	J. Th.	E. S.	Average.
<i>a</i>	228	284	337	283
<i>b</i>	292	306	357	318
<i>c</i>	275	267	366	303

Average of all = 301 c.c. per minute, which is equivalent to 1.46 calories per minute or 2100 calories per day.

The values recorded are distinctly high, appreciably higher than in normal men of the same weight. For example in C.N.H.L., who is both a heavier and a more vigorous subject than the three experimented on, the resting oxygen intake is about 250 c.c. per minute in a number of experiments. Whether there is any meaning in this greater metabolism we cannot say: we have not examined the matter specifically. It is obvious, however, from Table 5, that the recent administration, or the absence, of insulin produced no obvious result (on the average) in the resting oxygen intake. We have not measured the true basal metabolism of these subjects, but the resting metabolism is appreciably greater than in normal men under similar conditions. Such figures as we have sometimes recorded (*e.g.* for E.S. lying at rest, over 400 c.c. per minute) would be open to grave suspicion, were it not for the fact that the resting respiratory quotient shows very constant values in spite of this wide range in the figures of the gaseous exchange. The average value for all these subjects is about 300 c.c. per minute, which with a respiratory quotient of 0.76 implies the *oxidation of about 100 gms. of carbohydrate per day* for the resting individual.

The most important point brought out by the experiments of Table III concerns the respiratory quotient of the excess metabolism. For this purpose the experiments have been divided into three categories, (*x*), (*y*) and (*z*): (*x*) = insulin in the last 17 hours: exercise, E.S. up to 8 minutes, J.Th. up to 3 minutes, F.T. up to 3½ minutes; (*y*) = insulin in the last 17 hours: exercise, F.T. 5 to 8 minutes, J.Th. 5 to 8 minutes; (*z*) = all subjects without insulin 20 to 30 hours: all periods of exercise 0.5 to 8 minutes. The subject E.S. is obviously in a higher category than the others: in physique and in general appearance he seems a healthy robust man; he regularly follows his occupation and indulges in outdoor sport of not too vigorous a nature. He approximates most closely to the normal man, indeed a normal individual living on a fat diet yields similar results to his.

The three categories (*x*), (*y*) and (*z*) are obviously to some degree arbitrary : they do, however, represent in general :—(*x*) exercise of short duration with recent administration of insulin ; (*y*) exercise of longer duration with recent administration of insulin ; (*z*) all durations of exercise in the absence of a recent administration of insulin. The results of this classification are very striking. In category (*x*) the average value for the respiratory quotient of the excess metabolism in 17 experiments is 0.99 as compared with a previous average respiratory quotient of 0.76 : *clearly a diabetic subject with a recent administration of insulin, undertaking exercise of moderate duration and intensity, even on a diet largely consisting of fat, oxidises nothing but carbohydrate to meet the energy expenditure of his muscles.* This entirely confirms the conclusions of Furusawa in his studies of normal men on a fatty diet, and extends them to the case of a diabetic with a recent administration of insulin. In category (*y*), exercise of longer duration with a recent administration of insulin gave a respiratory quotient of the excess metabolism of 0.85 as compared with a resting respiratory quotient of 0.76. This again is analogous to Furusawa's results on normal men on a fatty diet, undergoing exercise of longer duration. It implies that, although at rest only 15 per cent. of the energy was being derived from carbohydrate, the excess energy expended as the result of 5 to 8 minutes of exercise was derived, 50 per cent. of it from carbohydrate, 50 per cent. from fat. In category (*z*), exercise of short, moderate or long duration without recent administration of insulin, the average respiratory quotient of the excess metabolism is 0.80 as compared with a previous average resting value of 0.76. This implies that, whereas at rest 18 per cent. of the energy was being derived from carbohydrate, the excess energy due to muscular exercise was derived from carbohydrate to the extent of about 32 per cent.

Burn and Dale (9) have proved that eviscerated preparations of cats, either normal or previously rendered diabetic by complete pancreatectomy, show a respiratory quotient of unity which remains practically unchanged under the action of insulin. Obviously the muscles, even of a completely diabetic animal, are able to oxidise carbohydrate alone. In our experiments, as in Furusawa's, human subjects with a sufficient supply of exogenous or endogenous insulin carry out exercise of short duration at the expense only of the oxidation of carbohydrate. When the exercise is prolonged, further processes are involved, which, it is difficult not to believe, imply the transformation of some other food substance to carbohydrate, which is so rendered available for the metabolism of the muscles. In the absence of insulin the respiratory quotient of the excess metabolism apparently is never unity,

however short the interval of exercise. Even so the values found show that about twice as large a proportion of the energy required for muscular exercise is being obtained from carbohydrate, as previously at rest.

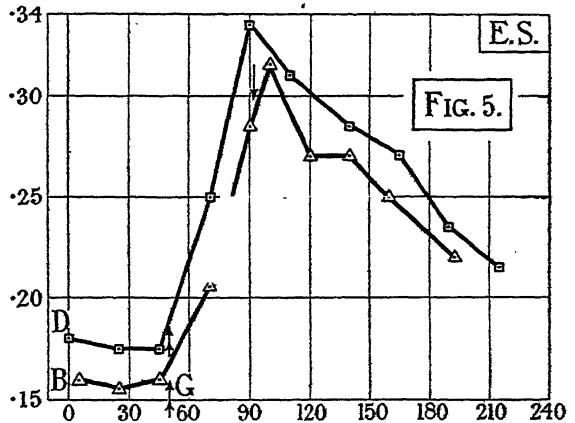
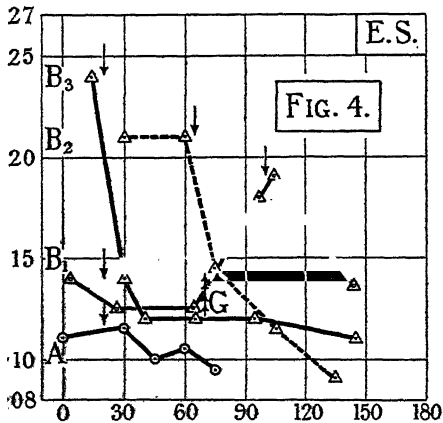
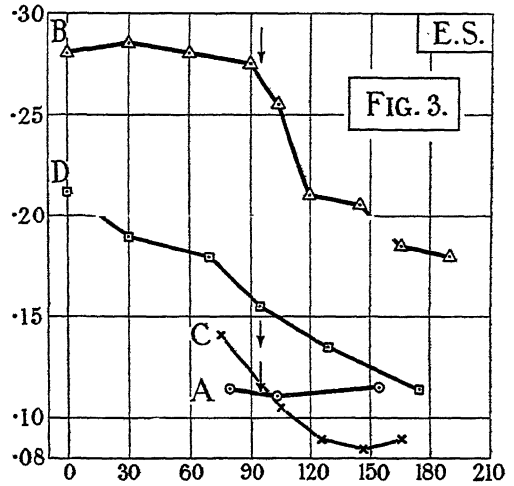
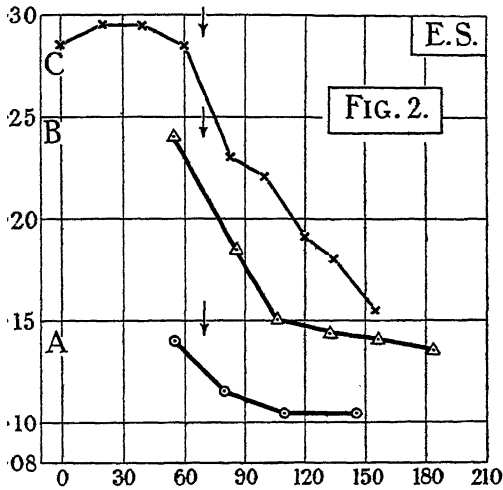
It would appear that the results found on diabetic patients undertaking exercise without insulin, represent merely an exaggeration and an extension of the phenomena observable on normal men on a fatty diet, or undertaking prolonged exercise. In the presence of insulin there is a sufficient store of carbohydrate in a form readily available for use by the muscles. Only when the exercise is of short duration is there no necessity for this to be replaced. When the exercise is of longer duration it is restored by the transformation of some other substance before recovery is complete. In subjects without insulin for some time previously, the store of carbohydrate readily available for use by the muscles has been so depleted that even the most moderate muscular exertion is followed by a restoration of the carbohydrate utilised as the result of the metabolism of the muscles.

If, as some would suppose, the muscles can oxidise fat directly to supply the energy necessary for the recovery process in muscle, it is difficult to understand why diabetic subjects, obviously needing to conserve their carbohydrate, living on a diet poor in carbohydrate, and without insulin in some cases from 10 to 17 hours, should nevertheless (in exercise of short duration) proceed to oxidise *carbohydrate only* to supply the energy required. It is far simpler to suppose that the muscles themselves utilise carbohydrate only, both in the normal and in the diabetic organism, and that so far as concerns the muscles insulin is necessary only in order to maintain at a sufficiently high level the store of carbohydrate, in some readily available form, for use in the oxidations necessary for recovery.

Our subject, F.T., is the most severe diabetic of the three. His physique is not as good as that of the others, although recently he has improved greatly. It is interesting to record that this subject complains far more than the others of weariness after the longer periods of exercise. Furusawa and other observers have noted great weariness when exercising a normal subject after some days on a fat diet. This further emphasises our belief that these diabetic subjects were, in relation to muscular exercise, in the same case as normal subjects who had lived for some time on a diet free of, or poor in, carbohydrate.

Part III.—Observations on the Blood-Sugar.

Since exercise increases the utilisation of carbohydrate by the diabetic, and since variations occur in the extent of this utilisation with and without



FIGS. 2-5.—Effect of exercise on blood-sugar.

Subject E.S.—Ordinate = blood-sugar per cent. Abscissa = time in minutes.

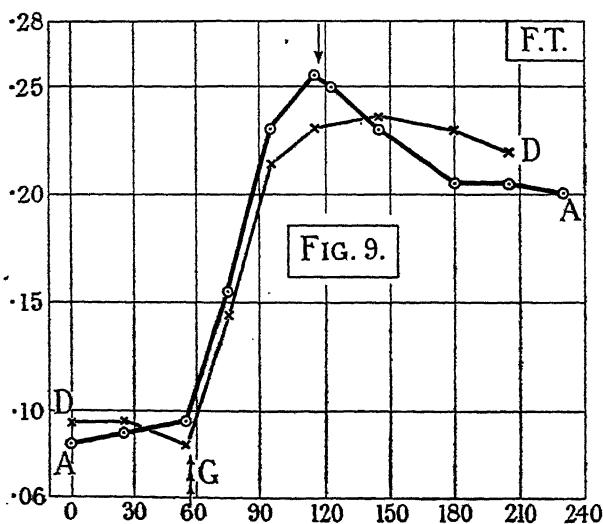
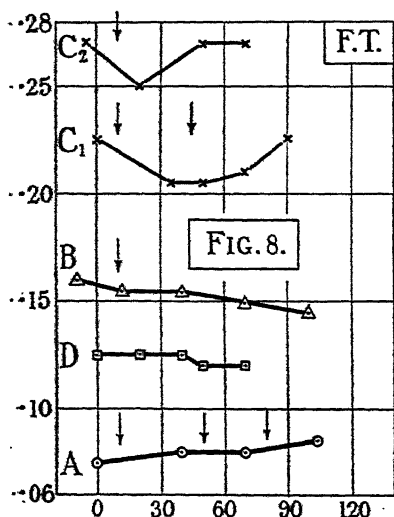
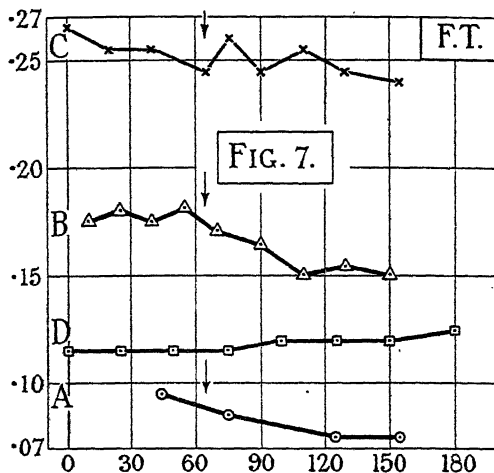
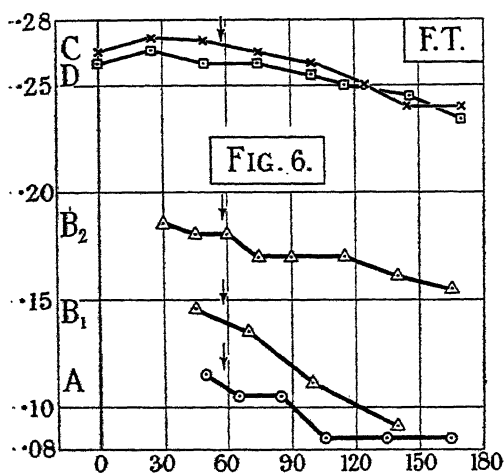
In all figures a vertical arrow denotes exercise undertaken, at 156 steps per minute (unless otherwise stated) for the period noted.

FIG. 2.—Exercise 8 minutes. Curve A.: Insulin $3\frac{1}{2}$ hours, food 3 hours before the experiment. Curve B.: Insulin $3\frac{1}{2}$ hours, food $2\frac{1}{2}$ hours before the experiment. Curve C.: Insulin 27 hours, food 2 hours before the experiment.

FIG. 3.—Exercise 5 minutes. Curve A.: Insulin $4\frac{1}{2}$ hours, food $3\frac{1}{2}$ hours before. Curve B.: Insulin $13\frac{1}{2}$ hours, food 2 hours before. Curve C.: Insulin $27\frac{1}{2}$ hours, food 2 hours before. Curve D.: The gradually falling blood-sugar of a diabetic at rest. Insulin 13 hours, food 2 hours before.

FIG. 4.—Curve A.: Exercise $3\frac{1}{2}$ minutes, at 210 steps per minute. Insulin $2\frac{1}{2}$ hours, food 2 hours before. Curve B₁: Exercise 104 seconds "all out." Insulin 13 hours, food $2\frac{1}{2}$ hours before. At G, 30 gms. of glucose taken by mouth. Exercise 101 seconds "all out." Curve B₂: Exercise 3 minutes. Insulin 15 hours, food $3\frac{1}{2}$ hours before. Curve B₃: Exercise $3\frac{1}{2}$ minutes at 210 steps per minute. Insulin 14 hours, food $2\frac{1}{2}$ hours before.

FIG. 5.—Two blood-sugar curves made under similar conditions. Insulin 13 hours, food $12\frac{1}{2}$ hours before. At G, in each case, 40 gms. of glucose taken by mouth. Curve D.: Subject at rest throughout. Curve B.: Exercise 5 minutes.



FIGS. 6-9.—Subject F.T. Effect of exercise on blood-sugar. Ordinate = blood-sugar per cent. Abscissa = time in minutes.

FIG. 6.—Exercise 8 minutes. Curve A.: Insulin 5 hours, food $4\frac{1}{2}$ hours before. Curve B₁: Insulin 11 hours, food 5 hours before. Curve B₂: Insulin 10 hours, food 4 hours before. Curve C.: Insulin 23 hours, food $4\frac{1}{2}$ hours before. Cf. D. Curve D.: Rest throughout. Insulin 23½ hours, food $4\frac{1}{2}$ hours before.

FIG. 7.—Exercise 5 minutes. Curve A.: Insulin 5½ hours, food 5 hours before. Curve B.: Insulin 10 hours, food $4\frac{1}{2}$ hours before. Cf. D. Curve C.: Insulin 23 hours, food $4\frac{1}{2}$ hours before. Curve D.: Resting control. Insulin 10 hours, food 4 hours before.

FIG. 8.—Various periods of exercise. Curve A.: Exercise $\frac{1}{2}$, 1 and 3 minutes. Insulin 6 hours, food $5\frac{1}{2}$ hours before. Curve B.: Exercise 100 seconds "all out." Insulin 11 hours, food 5 hours before. Cf. D. Curve C₁: Exercise $\frac{1}{2}$ and 1 minute. Insulin 23 hours, food 3 hours before. Curve C₂: Exercise 3 minutes. Insulin 24 hours, food 5 hours before. Curve D.: Resting control. Insulin 10 hours, food 4 hours before.

FIG. 9.—Two experiments performed at the same hour on consecutive days. At G, 40 gms. of glucose were ingested in each case. Insulin $4\frac{1}{2}$ hours, food 4 hours before. Curve D.: Subject at rest throughout—glycosuria persisted. Curve A.: Exercise .5 minutes. Urine sugar-free at end of experiment.

exogenous insulin, it was obviously desirable to ascertain whether corresponding variations occurred in the blood-sugar.

In experiments where insulin had been injected within the past six hours, and the blood-sugar was low to begin with, exercise had little or no observable effect on its concentration: it remained low. If the blood-sugar was not high, but above 0.10 to 0.12 per cent., exercise lowered it rapidly to below 0.10 per cent. In none of our experiments where the initial blood-sugar was low did exercise bring on *symptoms* of hypoglycæmia, although it will be seen below that exercise undertaken under such conditions, while there was any excess of insulin in the tissues, would cause a rapid lowering of the sugar concentration in the blood to the hypoglycæmic level.

Curve A, fig. 6, and curve A, fig. 7, represent graphically the results of an experiment in which exercise was undertaken within 6 hours of the injection of insulin, which occurred immediately before a meal.

When insulin is withheld from a diabetic patient for 10 to 17 hours, hyperglycæmia results from the ingestion and absorption of food, due to the moderate quantity of insulin in the tissues. This hyperglycæmia from absorption of carbohydrates rises to a maximum in 1 to 2 hours and then slowly decreases. This fall in the blood-sugar concentration is slower the more diabetic the patient is, that is, the less endogenous insulin there is to assist in the metabolism of the glucose. This gradual and continued fall of blood-sugar, in a resting diabetic subject, to whom insulin had not been given for 13 hours but who had partaken of food two hours previously, is demonstrated in curve D, fig. 3.

The effect of exercise under these conditions is to bring about a rapid fall in the blood-sugar level, *i.e.*, to produce rapidly what would occur gradually at rest. It is not likely that exercise should cause any great increase in the production or liberation of insulin by a deficient pancreas, and what apparently happens is that the restorative and oxidative processes are quickened with the insulin still available. The blood-sugar falls to a level ranging between 0.07 and 0.11 per cent. or higher, depending on the severity and length of the exercise, as well as on the height of the initial hyperglycæmia. Curve B, fig. 3, embodies the result of such an experiment, where the blood-sugar was watched for $1\frac{1}{2}$ hours before the exercise was undertaken. During this preliminary period the blood-sugar was practically stationary with a slight tendency to fall. Exercise caused an abrupt fall, after which the gradual resting fall recurred.

The subjects varied as regards the time interval, following the injection of insulin, during which the above effect of exercise was shown. At the end of

10 hours from the last injection of insulin, F.T., who received three doses of insulin totalling 50 units per diem, sometimes showed a fall as the result of exercise, and at other times no effect was produced beyond that which would have occurred at rest. Curve B₁ and B₂, fig. 6, demonstrate the results of two such experiments, the former showing a fairly rapid fall, the latter only the ordinary slowly falling curve of a diabetic at rest. In order to show that in this subject, F.T., a rapid fall of blood-sugar would occur had he an adequate supply of insulin in his body, he was given a weighed amount of glucose following his usual amount of insulin with a meal a few hours before. On one day he was given 40 gms. of glucose by mouth, and the blood-sugar was determined for 2½ hours following the ingestion, and for one hour previous to it. Two days later the experiment was repeated under similar conditions, and when, judging from the previous experiment, it was expected that the blood-sugar had reached its maximum point, exercise was undertaken and the effect on the blood-sugar noted. Curves A and D, fig. 9, represent the results obtained. The difference produced by exercise is striking, so that even in the severe diabetic the rapid fall of blood-sugar following exercise can be demonstrated, provided that there is adequate exogenous insulin in the tissues.

The subject E.S. showed the effects just described even when insulin had been omitted for 24 hours. His diabetes is not so severe as that of F.T., and presumably his production of endogenous insulin is greater. In F.T., when insulin had not been given for 24 hours, exercise had little effect on the concentration of glucose in the blood; curves C and D, fig. 6, representing experiments on F.T., are practically identical. In these insulin had been withheld for 23 hours, food being taken as usual. Curve C shows the behaviour of the blood-sugar before and after 8 minutes of exercise, curve D the behaviour during rest throughout.

An attempt was made in the subject E.S. to show that the rapid fall of blood-sugar following exercise occurred only when he had available insulin in his tissues. He was given a large dose of glucose when insulin had been omitted for 13 hours. It was hoped in this way to use up most of the insulin available in his tissues. Under similar conditions then in two experiments the blood-sugar was studied, following the ingestion of 40 gms. of glucose, (a) during rest throughout, and (b) before and after 5 minutes of exercise undertaken at the supposed height of the hyperglycæmia. The absence of any noticeable difference is seen in fig. 5. After the exercise the blood-sugar first rose, then fell abruptly, then followed a course practically parallel to that under resting conditions.

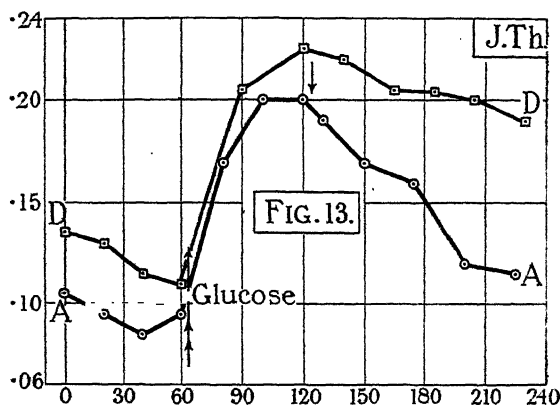
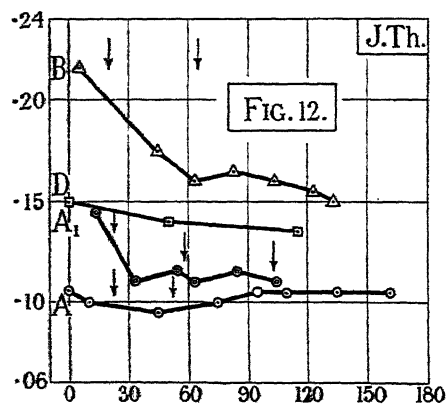
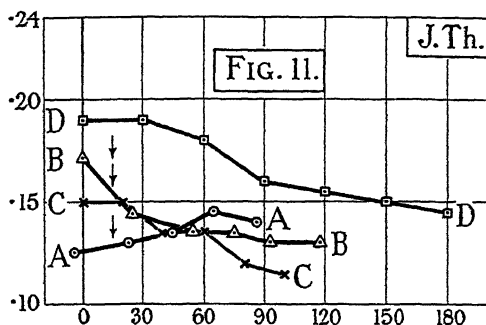
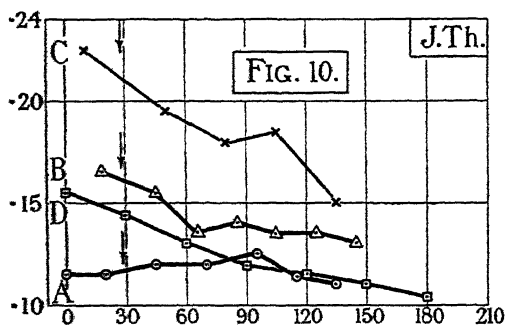
Exercise, therefore, in the diabetic, with an adequate supply of insulin, causes an increase in the amount of glucose disappearing from the blood, over and above that which would be removed with the same subject at rest for the same period of time. The question obviously arises, can this excess disappearance from the blood be attributed to the combustion of carbohydrates by the muscles, as calculated from the excess oxygen used as a result of the exercise?

To answer this question certain calculations are necessary, which are liable to possible objections, which will be considered first. (1) It is difficult to assess accurately the amount which the blood-sugar would have fallen during each experiment if the subject had remained at rest: for the rapidity and the amount of the fall vary with the initial height of the blood-sugar concentration, with the quantity of insulin in the tissues, and with the time interval from the last meal. (2) We can calculate from the blood-sugar only the net result of two processes which are going on continually, namely, the removal of sugar from the blood and the influx of sugar to the blood from the glycogen store. We have little idea of the actual amount of influx and outflow in this balance, and these may well be different during rest and during and following exercise. In any case it would seem unlikely that the influx of sugar into the blood from the glycogen stores is less during exercise and recovery with a rapidly falling blood-sugar, than at rest. Any figure, therefore, which we may calculate from the resting condition for the excess removal of sugar from the blood during and following exercise will very probably err on the small side. (3) It is necessary to postulate that the sugar is in diffusion equilibrium throughout the fluid tissues of the body. This may or may not be accurately true, though as a general statement it would be probable. We shall assume for the purposes of calculation that half the body-weight is fluid.

Acknowledging, then, the problematical nature of these calculations, it is of interest to ascertain if the excess oxygen used as a result of the exercise, with the observed respiratory quotient, would be sufficient to burn completely an amount of glucose equal to that which disappears in excess from the blood.

In the calculation it has been assumed that 1.34 gms. of glucose are oxidised per litre of oxygen used in the combustion of carbohydrate; also, for the sake of simplicity, what is very nearly true, that the same amount of energy is derived from the use of a given amount of oxygen, either in the com-

bustion of carbohydrate or of fat. The following results have then been obtained. (See Table VI.)



FIGS. 10-13. Subject J.Th. Effect of exercise on blood-sugar. Ordinate = blood-sugar per cent. Abscissa = time in minutes.

FIG. 10.—Exercise 8 minutes. Curve A.: Insulin $4\frac{1}{2}$ hours, food 4 hours before. Curve B.: Insulin $10\frac{1}{2}$ hours, food 4 hours before. Cf. D. Curve C.: Insulin 29 hours, food 4 hours before. Curve D.: Resting control. Insulin $10\frac{1}{2}$ hours, food 4 hours before.

FIG. 11.—Exercise 5 minutes. Curve A.: Insulin 5 hours, food 4 hours before. Curve B.: Insulin 17 hours, food $4\frac{1}{2}$ hours before. Cf. D. Curve C.: Insulin 29 hours, food 4 hours before. Curve D.: Resting control. Insulin 16 hours, food $3\frac{1}{2}$ hours before.

FIG. 12.—Various periods of exercise. Curve A.: Exercise $\frac{1}{2}$, 1 and 3 minutes. Insulin 5 hours, food $4\frac{1}{2}$ hours before. Curve A₁: Exercise $\frac{1}{2}$ and 1 minute. Insulin $5\frac{1}{2}$ hours, food 5 hours before. Cf. D. Curve B.: Exercise 1 and 3 minutes. Insulin $16\frac{1}{2}$ hours, food 5 hours before. Curve D.: Resting control. Insulin 4 hours, food $3\frac{1}{2}$ hours before.

FIG. 13.—Ingestion of 30 gms. of glucose at G. under similar conditions. Insulin $3\frac{1}{2}$ hours, food 3 hours before. Curve D.: Subject at rest. Curve A.: Exercise 5 minutes.

Table VI.—Fall in Total Sugar Content of Body, as calculated (a) from the Oxygen Intake and the Respiratory Quotient of the Excess Metabolism of Exercise, and (b) from the Fall in the Blood-Sugar on certain Assumptions given in the Text.

E.S.—Weight (average) 68 kilos.

R.Q. of Excess Metabolism.	Percentage of Energy from Carbohydrate.	Excess O ₂ in litres noted.	Excess O ₂ used in Carbohydrate Combustion.	(a) Equivalent amount of Glucose Oxidised, in Grammes.	Excess fall in Blood-Sugar, per cent.	(b) Excess removal of Glucose in Body Fluid, in Grammes.
1.16	100	7.3	7.3	9.8	0.055	18.7
1.09	100	9.2	9.2	12.4	0.09	30.5
0.86	52	32.0	16.7	23.0	0.075	25.5
0.90	66	18.7	12.2	16.0	0.07	23.8
1.09	100	8.9	8.9	12.5	0.105	35.7
1.04	100	26.9	26.9	36.0	0.06	20.4
Total				110		155

J.Th.—Weight, 54 kilos.

0.95	83	3.8	3.15	4.2	0.04	10.8
0.91	70	12.9	9.0	12.0	0.025	7.0
0.86	52	17.8	9.2	12.4	0.04	10.8
Total				29.0		29.0

These figures can be calculated only for some of the experiments, where there was a fairly rapid fall in a high blood-sugar. In the case of E.S. the excess removal of glucose from the body fluids is about 40 per cent. greater than the amount of glucose burnt as calculated from the respiratory exchanges. In J.Th. these quantities are about equal. Whether the difference observed in E.S. is due to an error in the various assumptions made, as it may well be, or whether it represents a genuine excess removal of glucose from the body fluids over and above the amount actually burnt as the result of exercise, it is not possible as yet to say. In any case it is obvious that the removal of glucose produced by exercise may be accounted for, in large part, by its combustion as the result of the exercise.

Part IV.—(A) The formation and oxidation of ketones. (B) The urinary nitrogen. (C) The sugar excretion in the urine.

(A) *Ketones.*—In all the experiments the urine has been examined for ketones by the rough colorimetric test described previously, and when the

exercise was of long duration the ketones have been estimated in the blood. In general, the results of the two types of observation agree, although in some cases we have found the urine to be acetone-free at a time when the blood showed an appreciable quantity of acetone, large enough in other experiments to have produced a reaction in the urine. Here, obviously, we are dealing rather with the question of ketone excretion by the kidney, which we have not investigated further.

The quantity of ketones found in the blood has been relatively small, as was to be expected with patients receiving adequate insulin and a fair moiety

Table VII.—Urinary Ketones in Relation to Muscular Exercise.

Subject F.T.

Hours since last Insulin.	Duration of Exercise, Minutes.	Blood Ketones, Mgms. per 100 c.c.			Urinary Ketones.		
		Before Exercise.	At end of Exercise.	At end of Recovery.	Before Exercise.	During Recovery.	At end of Recovery.
5	8	5.5	4.0	1.5	±	±	Nil
4	Resting with ingestion of glucose	23.5	—	8.0	+ ±	±	±
10	5	7.0	—	1.0	+	±	Nil
	with glucose.						
10	8	Trace	Nil	Nil	+ ±	+ ±	+
10	5	Nil	—	Trace	+	+	+ ±
10	5	9.0	7.0	5.5	+ ±	±	±
10	Resting	6.5	—	3.0	+	+	+
22	8	12.0	9.0	5.5	++	++	++
23	8	1.0	5.5	10.5	++	±	±
23	5	6.5	—	6.5	+ ±	+	+
23	Resting	4.5	—	4.5	+ ±	+ ±	+ ±

Subject E.S.

13	8	2.5	1.0	0.5	Nil	Nil	Nil
13	5	12.5	10.0	7.5	+	Nil	Nil
27	5	Nil	Nil	Nil	Nil	Nil	Nil
27	8	Trace	Nil	Nil	Nil	Nil	Nil
13	Resting	—	—	—	±	±	±

Subject J.Th.

4	8	7.0	4.0	2.0	±	—	±
5	5	1.5	—	3.0	Nil	—	±
16	8	9.5	7.0	1.0	+	+	Nil
17	5	4.0	—	5.0	+	—	±
29	5	15.5	—	9.5	+++	+ ±	±
29	8	6.0	5.5	—	+	—	Nil
16	Resting	—	—	—	±	+	+ ±

of carbohydrates in their diet. The amount of ketones in the blood of diabetics varies throughout the day, depending upon the diet, being increased after a meal with a high fat content. In order to be able to compare any results of exercise on the production and elimination of ketones the experiments must be performed at the same time of day and must bear the same relation to a similar meal.

Himwich, Loebel and Barr (3), in diabetic patients not actually receiving insulin at the time of the experiment, found little alteration in the acetone in the blood following exercise: variations in either direction occurred. While the changes which we have observed in the acetone in the blood of subjects who were receiving insulin are small, still on the whole they point clearly in one direction. An initial ketosis, when shown by our subjects at rest, either persisted or decreased slowly during the time of the experiment. When there were available supplies of insulin in the tissues exercise quickly abolished or lessened the ketones in the blood and urine. In the experiments for which curve A, fig. 6, gives the result of the blood-sugar estimations, the blood ketones fell from 5.5 mgms. to 1.5 mgms. per 100 c.c. Such amounts are small, but all the results show a decrease under similar conditions, and this may be of some significance.

In some of these experiments on F.T. when he had been without an injection of insulin for 23 hours, exercise produced a definite increase in the ketones in the blood. This result, however, is not invariable. In the experiments for which curve C, fig. 6, gives blood-sugar results, the blood ketones increased from 1 to 10.5 mgms. per 100 c.c. as a result of 8 minutes' exercise when the subject had been without insulin for 23 hours. A similar experiment seven days later gave a constant acetone content of the blood of 6.5 mgms. per 100 c.c. during the three hours of the experiment, while a resting control ten days later under similar conditions showed a constant amount of ketones in the blood.

In the subject E.S. we were unable to demonstrate any increase in ketones following exercise: in his case exercise always tended to decrease a ketosis existing previously. The subject J.Th. was again intermediate between the other two, for just as exercise failed to produce an abrupt fall in his blood-sugar, so also did it fail to banish the ketones from his blood. A fall from 15 to 9 mgms. per 100 c.c. represents the usual magnitude, in this subject, of the fall in ketones as the result of exercise.

The general conclusion one would draw is that exercise in these subjects decreases the ketones in the blood, if and when there is an available supply of insulin in the tissues, the ketones presumably being burnt as the result of the

increased utilisation of carbohydrate. The fall in the blood-sugar and the decrease in the ketones seem to run more or less parallel. In some experiments, where exercise had little effect on the blood-sugar, insulin not having been given for over 20 hours, the ketonæmia increased or remained stationary.

(B) *Urinary Nitrogen*.—The controversy as to whether muscular work increases protein metabolism still continues. To mention only two views, Lusk (10) makes a categorical statement to the contrary, and Cathcart (11) comes to the conclusion that exercise does enhance protein metabolism. It is, however, generally accepted now that protein is not the source of muscular energy when carbohydrate and fat are available.

For such short periods of exercise as we employed it is evident that there could be no great increase in the metabolism of protein in muscle, certainly not sufficient to upset the general assumption that the protein metabolism need not be taken into account in interpreting the rise in the respiratory quotient as the result of exercise. We have not found it possible to watch the total nitrogen excretion of these subjects for 24 hours or longer, following on the exercise, as would be necessary for a proper comparison of the results with those of a resting day; the subjects were following their occupations and hospital control could not be exercised. In order to show, however, that the total nitrogen excretion was not greatly altered during the period of time covered by the experiments we estimated the total nitrogen excretion in the urine passed during that interval. As the time of day during which the experiments were performed was constant, and as it bore the same relation to a similar meal, a certain value may be attached to the result. For a really adequate study it would be necessary to exercise a more rigid control of the diet than is possible for ordinary diabetic subjects employing the usual gravimetric measurements of the constituents of their food.

The result of these measurements of the nitrogen excretion was as follows. When the time during which the urine was collected was taken into account, the excretion of nitrogen did not vary much from one experiment to another. While little can be deduced from our figures as they stand, they do seem to show that there is no appreciably increased protein breakdown during and following the exercise, at any rate of a magnitude which would require any modification of the preceding argument. There is indeed no reason to suppose that the diabetic in this respect is in any way different from a normal man. Table VIII gives, so far as they go, our experiments on this matter.

(C) *Sugar excretion*.—Glycosuria occurred in these patients when insulin had been withheld for varying periods; whenever the blood-sugar was caused

Table VIII.—Nitrogen Excretion during Period of Exercise and Recovery.

Subject.	Hours since last Insulin.	Interval, in minutes, of Urine Collection.	Duration of Exercise, in minutes.	Total Nitrogen Excretion (Grammes) during Experiment.
E.S.	13	165	8	3.5
E.S.	13	190	5	3.15
			with glucose	
E.S.	13	180	5	3.2
E.S.	14	170	4	3.15
F.T.	5	175	8	2.50
F.T.	4	200	Resting	3.5
			with glucose	
F.T.	4	200	5	3.5
			with glucose	
F.T.	10	170	8	3.0
F.T.	10	155	5	2.7
F.T.	10	220	5	3.72
F.T.	10	180	Resting	2.60
F.T.	23	180	Resting	2.85

to fall as the result of exercise, the glycosuria disappeared, thus confirming the blood-sugar observations. In those experiments in which the blood-sugar remained above the renal threshold for glucose (*e.g.*, F.T. without insulin for 23 hours) glycosuria persisted and in some cases definitely increased following the exercise. This may perhaps be attributed to an increased blood flow to the kidneys following the exercise.

Summary.

1. Muscular exercise in the diabetic individual appears to be accompanied by the same metabolic changes as in the normal. The curves of recovery oxygen intake and of carbon dioxide output are similar to those of normal men, both for mild and for more strenuous exertion. The increase in lactic acid in the blood attained values as high as in normal men performing similar exercise. There is no unusual delay in the time required for the removal of this lactic acid.

2. Exercise increases the combustion of carbohydrate in the diabetic. For short periods of exercise, with insulin administered during the last 17 hours, the respiratory quotient of the excess metabolism is unity, exactly as in normal men: for exercise of moderate duration the value of the respiratory quotient of the excess metabolism is intermediate, while for exercise of long duration it tends to fall towards that of the previous resting metabolism. In this respect the diabetic individual, with recent insulin on a diet poor in carbohydrate, behaves in a manner exactly similar to a normal man on a diet consisting mainly of fat.

The phenomena, however, are even more exaggerated than in such a normal individual.

3. In a diabetic individual without insulin for long periods the respiratory quotient of the excess metabolism is low, even for short periods of exercise.

4. The simplest way of regarding the matter is to suppose that a muscle, for its oxidative processes in recovery from exertion, uses carbohydrate only; that in the presence of insulin there are stores of carbohydrate in a form readily available for use by the muscle, and that a short interval of exercise does not sufficiently deplete these stores to render an immediate restoration necessary from other substances in the body. In the absence, however, of insulin these ready stores of carbohydrate have run low, so that exercise even of the shortest duration stimulates the metabolic processes of the body to restore the reserves of carbohydrate. In this respect three factors, (i) prolonged muscular exertion, (ii) a diet poor in carbohydrate, and (iii) the absence of insulin, produce the same effect, namely, a lowering of the respiratory quotient of the excess metabolism due to exercise. All these factors might be expected to deplete the stores of carbohydrate readily available.

5. The hyperglycæmia following ingestion of food in the diabetic is shown to be reduced rather abruptly by a bout of exercise. This result, however, occurs only when insulin is available in the body: in the absence of recently administered insulin the fall in blood-sugar on exercise is no more rapid than at rest.

6. An approximate calculation has been made, from the lowering of the blood-sugar concentration, of the amount of sugar which has disappeared from the whole body in excess of that lost in a resting interval of the same duration. The quantity so measured is of the same order of size as the amount of sugar burnt during the same interval, as calculated from the excess oxygen intake and the respiratory quotient. On the average the sugar which has disappeared appears to be rather larger than that which has undergone oxidation. The data available, however, for the calculations are rough, and it would seem possible that the disappearance of sugar, following exercise, may be attributed simply to the process of oxidation.

7. With a sufficient supply of insulin in the tissues of a diabetic a more complete combustion of fat takes place on exercise than at rest. Without available insulin exercise may at times result in an increased production of ketones.

We wish to express our appreciation of the help we have received from Prof. A. V. Hill and Prof. T. R. Elliott during this research.

APPENDIX.

Clinical Notes on the three Subjects.

E.S., aged 36. Decorator. Admitted to hospital March 19th, 1924. Discharged, April 28th, 1924.

Thirst, polyuria, loss of weight and indigestion had been noticed for two months. Weight on admission, 60·6 kilos. Urine passed in 24 hours on ordinary diet measured 3,060 c.c., and contained 4 per cent. glucose, acetone +++ and diacetic acid ++.

The patient was starved until his urine was sugar-free, and then a gradually increasing diet was given until, at the time of discharge, his diet was 2090 calories, containing carbohydrate, 69 gms.; protein, 77 gms.; fat, 167 gms. To keep the urine sugar-free and his blood-sugar fluctuating within normal limits, 30 units of insulin a day were necessary. It was given in two doses of 20 and 10 units each. The first experiments were started 3 weeks after discharge from hospital, and observations were continued until March, 1925, when his weight was 69·8 kilos. E.S. leads an active life, and feels and looks a fit man.

J.Th., aged 16. Admitted to hospital, September 4, 1924. Discharged, December 6, 1924.

Thirst, polyuria and loss of weight present for two months. Weight on admission, 49 kilos. On ordinary diet the urine voided in 24 hours measured 1800 c.c., and contained 6·25 per cent. glucose and acetone ++.

Starvation rendered the urine sugar-free in two days. Diet increased in stages to carbohydrate, 47 gms.; protein, 58 gms.; fat, 167 gms. = 1,920 calories. 25 units of insulin in 2 doses of 15 and 10 units each were given to keep the blood-sugar within normal limits. Observations on the effect of exercise were made during his stay in hospital. Ten experiments were done in June, 1925. Weight on discharge from hospital = 54 kilos. Weight in July, 1925, 69·8 kilos. This lad is growing fairly rapidly and looks robust and fit.

F.T., aged 21. Clerk. Admitted to Hospital, April 30, 1923. Discharged June 5, 1923.

Thirst, polyuria and loss of weight present for 18 months. He was known to have diabetes mellitus during this time and had received treatment by restriction of diet. Weight on admission to hospital = 55·1 kilos.

Three months before the experiments began the diet was increased to 1840 calories, containing carbohydrates, 52 gms.; protein, 92 gms.; fat, 140 gms. The insulin was also increased to 50 units a day given in three injections of 20, 10 and 20 units each. Insulin had been given for 12 months before the experiments began. His weight in June, 1924, when the observations commenced was 57·1 kilos. His blood-sugar has remained consistently low, and any increase in strength of a batch of insulin was immediately shown by hypoglycæmic symptoms. His general condition has improved greatly and his capability of doing exercise has also increased. Weight, July, 1925, 63·5 kilos. Experimental observations extended over 12 months.

REFERENCES.

1. Hill, Long and Lupton. 'Roy. Soc. Proc.' B, vols. 96 and 97 (1924).
2. Furusawa. 'Roy. Soc. Proc.' B, vol. 98, p. 65 (1925).
3. Himwich, Loebel and Barr. 'Journ. Biol. Chem.', vol. 59, p. 265 (1924).

4. Doisey, Briggs, Weber and Koechig. 'Proc. Soc. Exp. Biol. and Med.,' vol. 22, p. 57 (1924).
 5. Maclean, H. 'Modern Methods of Diagnosis and Treatment of Diabetes and Glycosuria' (1922).
 6. Clausen. 'Journ. Biol. Chem.,' vol. 52, p. 263 (1922).
 7. Shaffer. 'Journ. Biol. Chem.,' vol. 5, p. 211 (1908-9).
 8. Van Slyke. 'Journ. Biol. Chem.,' vol. 32, p. 455 (1917).
 9. Burn and Dale. 'Journ. Physiol.,' vol. 59, p. 164 (1924).
 10. Lusk. 'Science of Nutrition,' p. 316 (1917).
 11. Cathcart. 'Physiol. Reviews,' vol. 5, p. 225 (1925).
-

A Closed-Circuit Heart-Lung Preparation.—Effect of Alterations in the Peripheral Resistance and in the Capacity of the Circulation.

By I. de BURGH DALY.

(Communicated by Prof. E. H. Starling, F.R.S.—Received November 12, 1925.)

(From the Physiological Institute, Cardiff.)

Investigations of "resistance" and "capacity" effects in relation to the control which they exert upon the vascular system have been carried out by many physiologists, notably by Weber (1), Volkmann (2), Donders (3), de Jager (4), Bayliss and Starling (5), and L. Hill (6, 7). According to Weber, the rise in arterial blood pressure due to arteriole vaso-constriction is caused by an increase in peripheral resistance and by a diminution in the capacity of the circulation. Bayliss and Starling (5) brought forward evidence in favour of this view and pointed out that the venous pressure was in part determined by a balance between these two factors. In opposition to Weber's view, L. Hill and Barnard (6) hold that capacity changes have no effect on the venous pressure since the vascular system is not filled to distension. In a later paper L. Hill (7) states that reduction in the capacity of the splanchnic area is of importance in so far as it increases the diastolic filling, and so the output of the heart, but that the mean hydrostatic pressure cannot be considered to contribute to this result. The part played by constriction of the veins in altering the venous and arterial

pressure is discussed in a paper by Connet (8) in which a full bibliography is given.

In a paper published in the 'Journal of Physiology' (9) I described a modification of Starling's heart-lung preparation, in which the blood circuit was converted to a closed system, so as to imitate more closely the conditions ruling in the animal body, while maintaining the various factors fully under control of the experimenter. The arrangement is shown in fig. 1. The blood from the aorta flows through a cannula placed in the brachio-cephalic artery (A), the velocity of flow being recorded by a Pitot tube (B) or by a Henderson's cardiometer. The peripheral resistance is regulated by a compressible finger-stall (D), a second resistance (D') being inserted for studying the effects of shunt circuits. The blood then passes to the venous reservoir (K) which consists of a rubber bag of approximately 250 c.c. capacity. The distal end of the venous reservoir is connected to the superior vena cava. A finger-stall (C) joined by a side tube to the arterial system represents the elasticity of the arterial system; this finger-stall and the venous reservoir K when enclosed in plethysmographs enable the experimenter to study alterations in the volume of the systemic circulation.

In all experiments dogs were used, under full anaesthesia with chloralose, 1 decigram per kilo. body-weight being injected intravenously.

The Mean Systemic Pressure (Weber's sense).

Weber defined the mean systemic pressure in his artificial circulation mode as the pressure which obtained in the system when the fluid was at rest, and the hydrokinetic pressure as the pressure measured at any given point when the fluid was circulating. The hydrokinetic mean pressure was obtained by summing the pressure at each unit of length throughout the system and dividing by the number of units.

In the closed-circuit heart-lung preparation, the mean systemic pressure was taken in five experiments in the following manner. When the heart had stopped, the artificial resistance was lowered to zero and the pressures in the right and left auricles taken after an interval of five to ten minutes. There was always a slight difference in pressure between the two auricles, but in no experiment did it amount to more than 0.5 cm. of saline. The mean of the two readings was taken to be the mean systemic pressure in Weber's sense. In the five experiments these values were 12, 9, 2 and 8 cm. of saline, measured from the level of the right auricular appendix. In all probability, these figures

do not represent the true mean systemic pressure under working conditions, for as L. Hill (7) points out, the capacity of the vascular system in the intact animal after the circulation has been arrested will be far from normal. Furthermore, changes in the calibre of the blood-vessels in different parts of the system will cause a pressure and a blood redistribution. The same criticism applies to the measurements of the mean systemic pressure in the closed circuit preparation, as the capacity of the heart chambers, of the coronary vessels and pulmonary circulation will undergo alterations after cardiac arrest. The lowering of the artificial resistance brings the aortic and right ventricular pressures to the same level, so that only the pulmonary circulation will be responsible for any inequality of pressures in the system when the heart is stopped.

Weber maintained that the mean systemic pressure was equal to the mean hydrokinetic pressure. Calculations of the mean hydrokinetic pressure based upon the assumption that the lungs would contain $\frac{1}{5}$ th of the total body blood (Spehl, 10) of the intact animal from which the preparation was made, gave values of 15–20 cm. saline. The arterial and venous pressures were taken as those generally pertaining with a moderate cardiac output, and the body blood as 7 per cent. of the body-weight (Harris, 11). The mean systemic pressure calculated in this manner is probably nearer the true value under working conditions than that taken after arrest of the circulation.

Effect of Alterations in the Capacity of the Systemic Circulation.

The capacity was varied by allowing blood to flow into a collapsed finger-stall of known volume connected to the system by a side tube or by squeezing the finger-stall when already filled with blood. Experiments showed that a diminution in capacity of the systemic circulation produced the same results qualitatively and quantitatively as injection of blood into the circulation (9), namely, an increase in all the pressures and in the cardiac output; whereas an increase in capacity had the same effect as withdrawal of blood from the system. Capacity diminution and blood injection raise the mean systemic pressure, but capacity increase and withdrawal of blood lower the mean systemic pressure, fig. 5. At 1, 10 c.c. of blood were injected; at 6, 15 c.c. were injected. The capacity of the circulation was diminished at 3 and at 8.

Effect of Increasing the Peripheral Resistance.

The peripheral resistance was raised by compression of the rubber tube in the glass jacket, fig. 1, D, in the usual manner, the small change in the capacity of the circulation at this point being neglected. The arterial and venous pressures and the ventricular volume showed alterations which depended upon the amount of blood which the arterial system could accommodate in response to the rise in arterial pressure. A rubber bag, connected to the

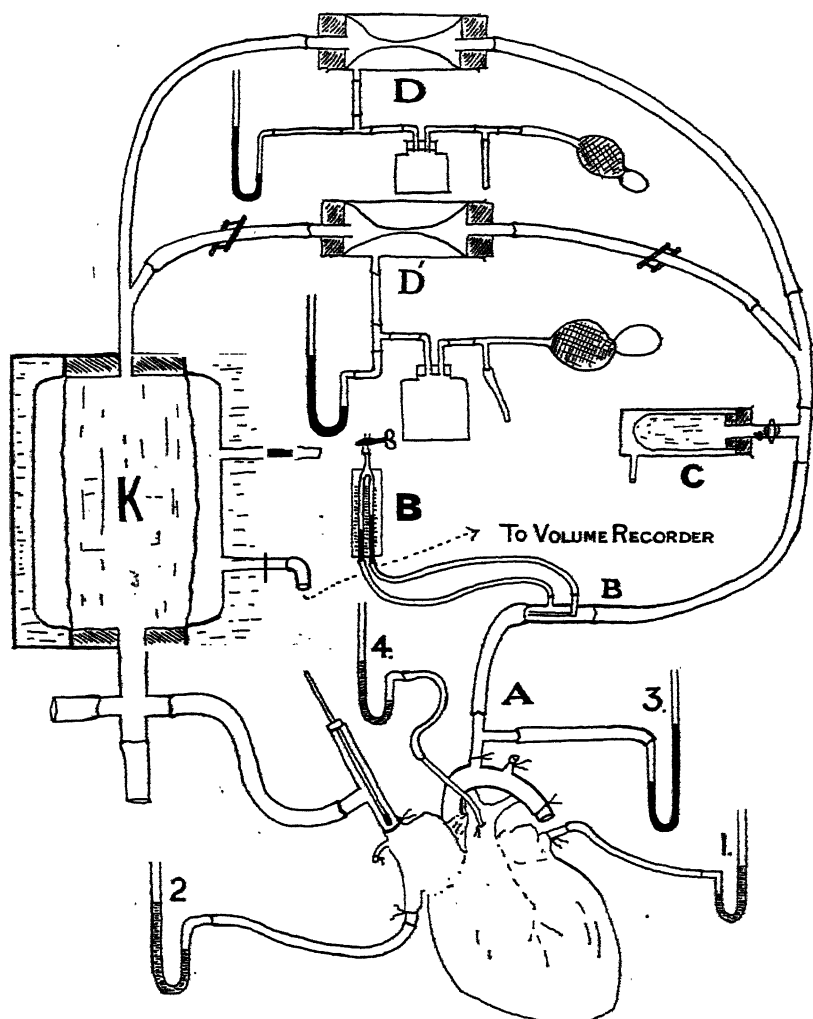


FIG. 1.—A, Cannula in brachio-cephalic artery. B, Pitot tube recorder. C, Elastic finger-stall. D, D', Artificial resistances. K, Rubber bag. 1, 2, 3, 4, Manometers recording pressures in the left auricle, right auricle, brachio-cephalic artery and pulmonary artery respectively.

arterial system by a side tube, having a small volume-elasticity coefficient ($\frac{\Delta p}{\Delta v}$) and a large initial capacity, will be able to accommodate more blood when the arterial pressure is raised than will a bag of smaller initial capacity and made of material having a larger volume-elasticity coefficient. The term "potential capacity increment" will be used to denote the extra amount of blood which the arterial system is capable of taking up; this will be relatively large in the young as compared with the old animal. The "potential capacity increment" of the arterial system was varied in three ways, and the experimental results fell into three groups, according to whether the capacity increment was large, moderate or small.

Group I.—Large "Potential Capacity Increment" of the Arterial System.

A rubber tube of 45 c.c. capacity, closed at one end and made of the same material as the venous reservoir, was connected by a side tube to the arterial system at C, fig. 1. On raising the peripheral resistance there occurred a fall of pressure in both auricles, a rise in the systemic pressure, a diminution in the output of the ventricles and a decrease in the mean ventricular volume as recorded by the cardiometer. Two experiments are given in Table I and a tracing in fig. 2.

The immediate effect of the peripheral resistance increase is to dam up some of the blood on the arterial side and reduce the flow into the right auricle; as a result, the pressure in the right auricle falls.

The fifth curve in fig. 2 (A.S.) is a plethysmograph tracing of the more distensible portion of the arterial system, an upward movement of the lever denoting an increase in volume. This tracing demonstrates the pooling of blood in the arterial system. As long as the peripheral resistance is kept high the blood remains re-distributed in this way, causing the diminished flow and the fall of pressure in the right auricle to persist; the output of the heart therefore decreases, and there is a fall of pressure in the left auricle. (Also in the pulmonary artery—see later.) Since the energy liberated during contraction of the heart muscle is dependent upon the initial length of the muscle fibre (Starling), the marked diminution of the diastolic volume of the ventricles indicates that the increase in energy required to overcome the raised peripheral resistance is less than the energy saved, due to the reduction in cardiac output. The conditions of the circulation are such that a rise in peripheral resistance reduces the total amount of work done by the heart.

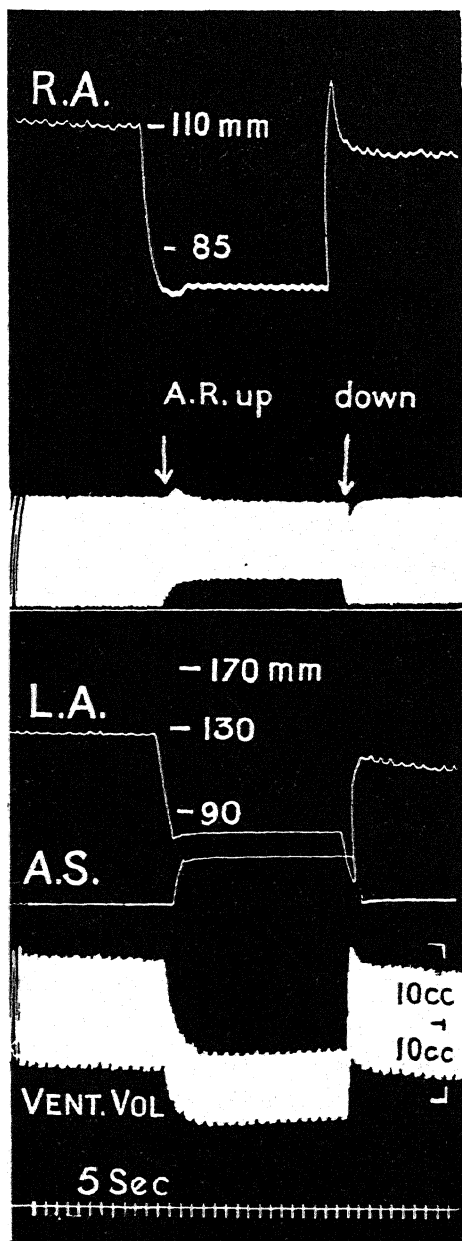


FIG. 2.—Large “potential capacity increment” of the arterial system. Peripheral resistance increased from 50 to 70 mm. Hg. A.S. = Plethysmograph of distensible portion of arterial system. In this and in all other tracings, systole causes a movement of the cardiometer lever downwards.

Table I.—Effect of Increasing the Peripheral Resistance. Large Potential Capacity Increment of Arterial System.

Experiment I.—Dog, 8.0 kilos. Heart weight 63 gms.

L.A.	R.A.	M.S.P.	A.R.	V.O.
—	96	68	55	6.4
—	50	95	85	2.8
—	100	60	50	6.8

Experiment II.—Dog, heart weight 53 gms. Temperature 34.0° C.

135	80	60	40	5.3
70	45	80	60	1.9
125	75	60	40	4.8

L.A.—left auricle. R.A.—right auricle, pressure in mm. saline. M.S.P.—mean systemic arterial pressure, mm. Hg. A.R.—artificial resistance, mm. Hg. V.O.—ventricular output, c.c. per beat.

Group II.—Moderate "Potential Capacity Increment" of the Arterial System.

It was recognised from the results obtained in Group I that if the output of the heart was to be maintained at anything approaching a constant value when the peripheral resistance was increased, either the capacity of the arterial system would have to be made smaller or its volume-elasticity coefficient larger. In Group II the capacity connected to the arterial side-tube was reduced to 25 c.c. and the bag used was the finger-stall, the pressure volume changes of which have been given in a previous paper (9). A rise in peripheral resistance lowered the venous pressures and the pulmonary arterial pressure, the ventricular output was diminished, but not so markedly as in Group I, and, in general, the mean volume of the ventricles showed only slight variations in either direction. The protocols of two experiments are given in Table II and tracings in figs. 3B, and 5 (2) (7).

The difference between these results and those obtained in Group I may be accounted for by the fact that the pooling of blood in the arterial system is less and the reduction in cardiac output less. In fig. 3 the diastolic volume of the ventricles remained approximately the same, the energy saved due to the diminished output being nearly counterbalanced by the extra energy required to overcome the increased peripheral resistance. The gradual decline of the cardiometer tracing, fig. 5 (6), is not altogether due to blood leakage. It has been found that after blood injection the mean ventricular volume generally

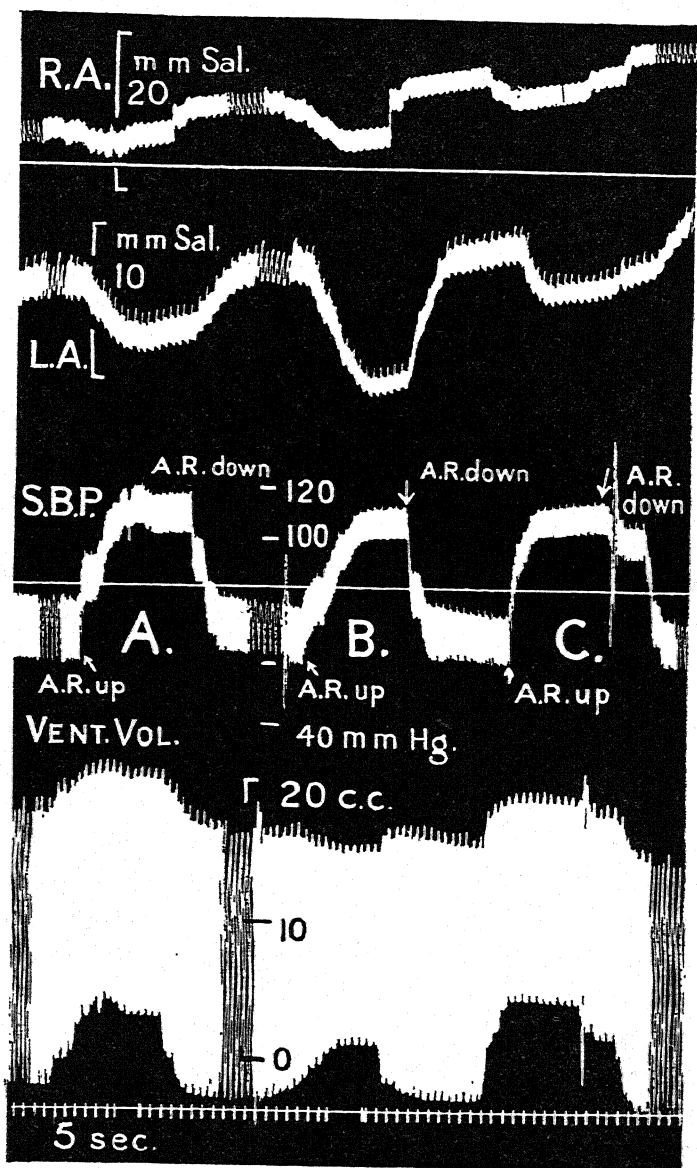


FIG. 3.—Effect of varying the “potential capacity increment” of the arterial system. Rigid resistance tube used. At A and C peripheral resistance raised without finger-stall in circuit; at B with finger-stall in circuit. The piston recorders registering the L.A. and R.A. pressures show a leakage effect. The initial values of the L.A. and R.A. pressures were 120 and 100 mm. saline respectively.

falls slightly after the initial rise, and that in some cases it is due to the temperature of the blood injected being slightly higher than that of the preparation. The time taken for the blood to be re-distributed and also the improved coronary flow, which results in the mean ventricular volume diminishing, although the total output remains the same, may take part in the effect.

Table II.—Effect of Increasing the Peripheral Resistance. Moderate Potential Capacity Increment of the Arterial System.

Experiment 1.—Dog. 8.0 kilos. Temperature 37.0° C. Rate 126–132 per minute.

L.A.	R.A.	M.S.P.	A.R.	P.A.	V.O.
50	95	75	56	20	10.0
45	82	80	90	12	9.2 A.R. raised.
50	95	62	44	20	9.8

Experiment 2.—Dog, 8.5 kilos. Ht. Wt. 85 gms. Temperature 36°.

70	45	63	40	18	4.0
66	40	80	70	15	3.2 A.R. raised.
70	45	62	40	18	3.9

P.A.—pulmonary arterial pressure, mm. Hg.

Four other experiments gave essentially the same results.

Group III.—Small "Potential Capacity Increment" of the Arterial System.

In order to obtain this condition, the finger-stall C was disconnected. In other experiments the cannula in the brachio-cephalic artery was joined by glass tubing to a metal tube with an adjustable bore, which took the place of the artificial resistance. The mechanism for altering the bore of this resistance tube was so constructed that its internal volume remained constant throughout the adjustment. With this arrangement the only distensible portion of the arterial system was the arch of the aorta. Tracings and protocols of experiments are shown in figs. 3 and 4 (A, C) and Table III.

Two other experiments gave the same result.

The results in Group III. differ from those obtained in Group II by several important features; these are brought out clearly in the tracing, fig. 3. With the smaller "potential capacity increment" of the arterial system, the fall of pressure in both auricles is less, and in some cases the pressure in the left auricle is raised (fig. 4); the systolic and diastolic volumes of the ventricles

are relatively larger and the percentage fall in output is less. The same results take place whether the peripheral resistance is increased to the same value or whether the arterial pressure is raised to the same value. Owing to the small distensibility of the aortic arch, only a slight redistribution of blood takes place and the call upon the heart muscle for extra energy to overcome the

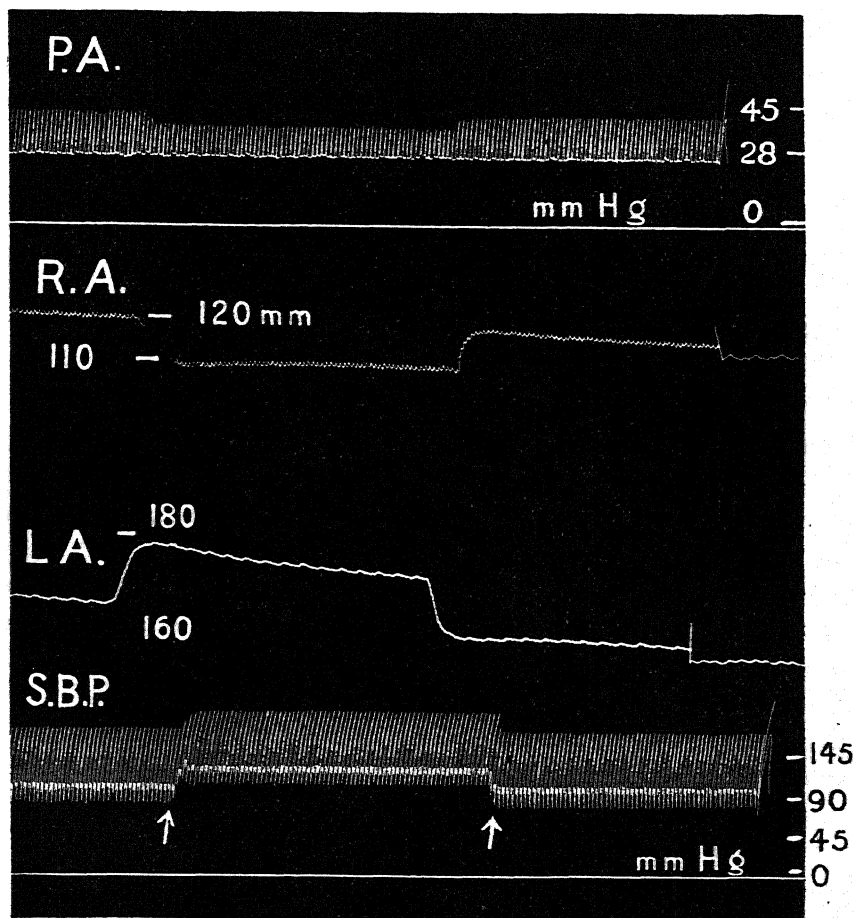


FIG. 4.—Small "potential capacity increment" of the arterial system. At first arrow A.R. raised from 70 to 125 mm. At second arrow A.R. lowered from 125 to 60 mm. Hg.

raised peripheral resistance is greater than the energy saved consequent upon the decreased output, the diastolic volume of the ventricles therefore increases.

In the experiments in Table III, in which the pressure in the left auricle rises, the rise may be accounted for by the increased diastolic pressure in the

Table III.—Effect of Increasing the Peripheral Resistance. Small Potential Capacity Increment of Arterial System.

Experiment 1.—Dog, 7.0 kilos. Heart weight, 59 gms.

L.A.	R.A.	M.S.P.	A.R.	P.A.	V.O.
110	35	108	70	25	—
130	30	128	85	19	— A.R. raised.
114	20	102	70	20	—

Experiment 2.—Dog, 6.5 kilos. Heart weight, 72 gms.

125	65	62	30	—	6.0
205	45	90	80	—	5.5 A.R. raised,
145	65	60	30	—	6.1

Experiment 3A.—Dog, 10.0 kilos. Heart weight, 125 gms. Rigid Resistance Tube.

132	110	72	—	—	10.0
122	100	108	—	—	8.8 A.R. raised.
130	110	74	—	—	9.8

left ventricle more than compensating for the usual fall, (Fühner and Starling, 12.) That this is the true explanation is supported by the fact that the greater the inflow and peripheral resistance or the more fatigued the heart, the more likely is the rise to appear. A rise in pressure in the left auricle will tend to produce "back pressure" effects in the pulmonary circulation, but the tracing in fig. 3 shows that the "back pressure" may not be sufficient to compensate wholly for the fall in pulmonary pressure due to the diminished cardiac output.

The mechanism of the alterations observed in the three Groups is the same, they only vary in degree. There is a transference of blood from the venous to the arterial system, resulting in reduced filling of the heart. The greater the "potential capacity increment," the greater is the reduction of the blood inflow to the heart for any given rise in peripheral resistance.

It is to be noted that the heart itself takes part in the changes of capacity.

Effect of Balancing the "Resistance" and "Capacity" Effects.

Since a rise in peripheral resistance in the closed circuit system is always accompanied by a fall of pressure in the right auricle and in the pulmonary artery, as well as a diminution in output, it follows, that if with a rise in peripheral resistance any one of these factors is to remain constant, or to be increased, there will have to be a compensatory reduction in the capacity of

the circulation or an injection of blood into the system. Either procedure will compensate for the blood drained away from the venous side of the system.

In order to obtain knowledge of the degree of capacity change or the amount of blood injection which was necessary to balance the fall of pressure or of cardiac output, due to peripheral resistance increase, four experiments were carried out in the following manner. The artificial resistance was raised in steps, and blood injected in measured quantities until the pressures or the ventricular output returned to their original values. The reverse procedure of injecting the blood first and then obtaining a pressure or an output balance by raising the artificial resistance was also adopted. Calculations were then made as to the amount of blood which had to be injected in order to balance the changes due to a 10 mm. Hg rise in peripheral resistance. It was found that this quantity depended upon whether it was the venous pressure, the pulmonary arterial pressure or the ventricular output which was to be balanced. In one experiment, 0.8, 7.0 and 4.0 c.c. were necessary for a balance of the changes in the left auricle, right auricle and ventricular output respectively; in a second experiment the values were 0.8, 3.0 and 3.2 c.c. for the same factors respectively; in a third experiment, 5.0, 6.0 and 7.0 c.c. for the left auricle, right auricle and pulmonary artery respectively, and in a fourth experiment, 8.0 c.c. for the change in the pulmonary arterial pressure. These results show that in order to counteract the change of pressure in the left auricle, a smaller quantity of blood injected is required than for the other changes. These experiments were not considered entirely satisfactory, as it was recognised that errors might be introduced owing to blood leakage from the system during the determinations.

Further experiments, therefore, were carried out, in which the peripheral resistance was raised and the various factors brought back to their original values by compression of the venous reservoir. By this method the determinations were more rapid and compensation for any slight leakage could be made. The actual amount of capacity change was not measurable, but the relative values of the various pressures were able to be observed. The procedure adopted is shown in the tracing fig. 5, which is a typical example. At (1) 10 c.c. blood injection, at (2) A.R. raised from 40 to 70 mm. Hg, at (3) compression of venous reservoir, at (4) compression released, at (5) A.R. lowered to 40 mm. Hg. A short interval then followed and at (6) 15 c.c. of blood were injected, at (7) A.R. raised to 80 mm. Hg, at (8) compression of venous reservoir, at (9) compression released, and at (10) A.R. lowered to 50 mm. Hg.

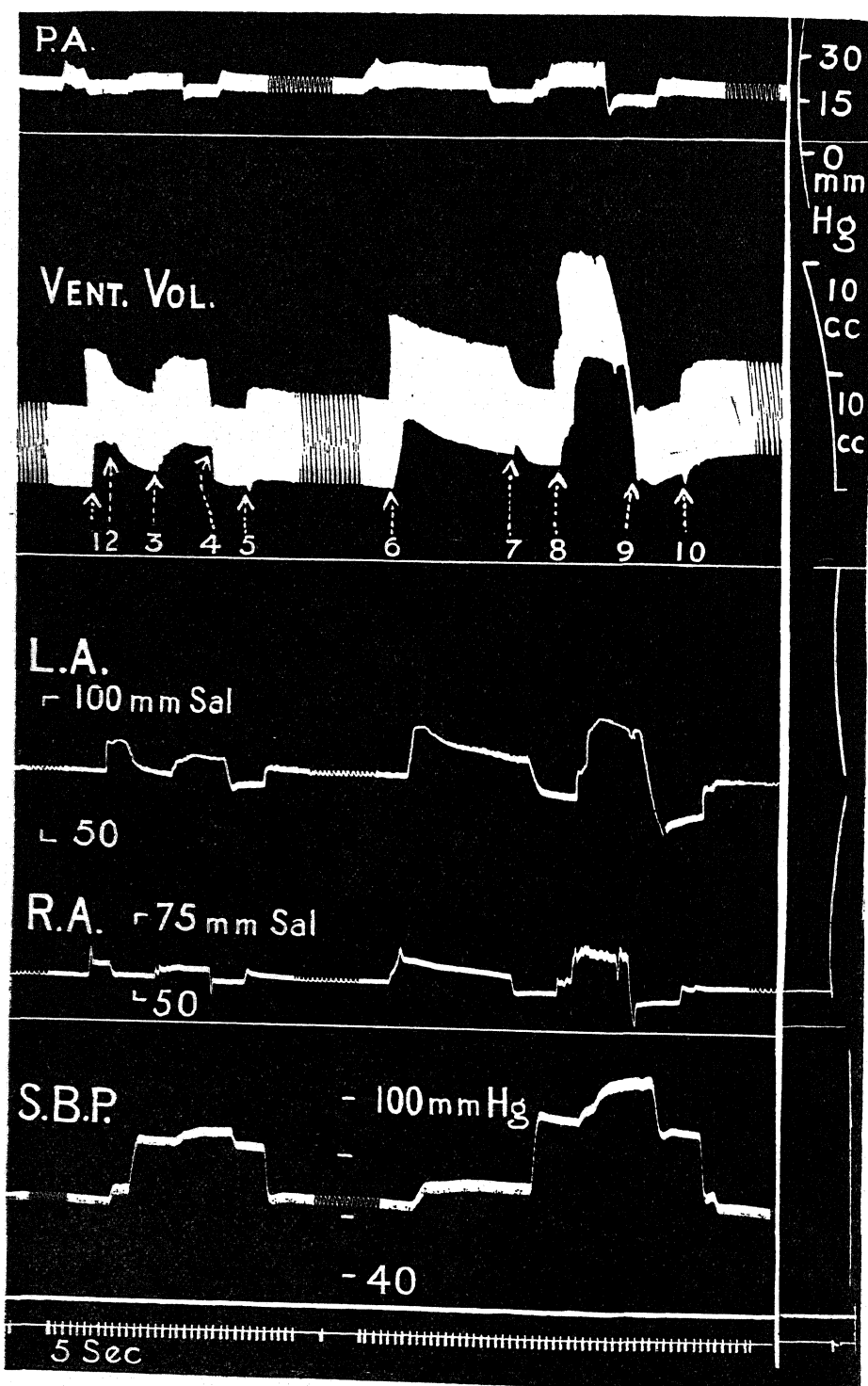


FIG. 5.—Moderate “potential capacity increment” of the arterial system. At (1) 10 c.c. of blood injected; at (2) A.R. raised from 40 to 70 mm. Hg; at (3) compression of venous reservoir, K; at (4) compression released; at (5) A.R. lowered to 40 mm. Hg; at (6) 15 c.c. of blood injected; at (7) A.R. raised to 80 mm. Hg; at (8) compression of venous reservoir to balance output; at (9) compression released; at (10) A.R. lowered to 50 mm. Hg.

Table IV gives the details of part of another experiment, and Table V shows the direction in which the pressures and cardiac output moved when one of them was balanced; they may be summarised as follows:—

When the arterial pressure is raised by an increase in peripheral resistance and by a decrease in the capacity of the circulation, so as to maintain the pressure in the left auricle constant, the pressures in the right auricle and pulmonary artery and the ventricular output are diminished. If by the same procedure the pressure in the right auricle is maintained constant, the pressure

Table IV.—Dog, 8·5 kilos. Ht. Wt. 85 gms. Temp. 36·0° C.

	L.A.	R.A.	M.S.P.	A.R.	P.A.	V.O.
(a)	70	55	80	40	26	—
	62	47	98	70	23	— A.R. up.
	75	57	103	70	26	— Resistance compression.
	70	50	100	70	24	— Compression readjusted.
	56	45	97	70	22	— Compression off.
	62	48	78	40	24	— A.R. down.
(b)	86	59	70	40	21	4·8
	74	53	92	80	15	3·4 A.R. up.
	98	68	106	80	22	4·8 Resistance compression.
	68	52	89	80	13	3·2 Compression off.
	80	56	63	40	18	4·1 A.R. down.

Table V.—Peripheral Resistance Increase Associated with Diminished Capacity of Systemic Circulation.

Effect upon.				Effect upon.			
L.A.	R.A.	P.A.	V.O.	L.A.	R.A.	P.A.	V.O.
L.A. balanced	-	-	-	P.A. balanced	+	0	0
	0	-	-		+	+	-
	0	-	-		+	+	-
	-	-	-		+	0	0
	0	-	-		+	+	0
0	-	-					

R.A. Balanced	+	0	0	V.O. balanced.	+	0	0
	+	0	0		+	+	+
	0	-	-		+	+	+
	-	-	-		+	+	+
	+	-	-				
	+	-	-				
	+	-	-				
	0	-	-				
	0	-	-				
+	-	-					

+ = increased.
- = decreased.
0 = no change.

From three experiments.

in the left auricle rises or shows no change, the pulmonary arterial pressure falls and the output of the heart diminishes. With the pulmonary arterial pressure kept constant the pressure in both auricles generally rises, and the ventricular output diminishes or is unchanged. When the ventricular output is maintained at the same level the left and right auricular pressures rise, and the pulmonary arterial pressure rises slightly, or may be unchanged. That is, in the majority of experiments, as the capacity is progressively decreased, the left auricular pressure first attains a balance, then the right auricular pressure, then the pulmonary pressure, and finally the ventricular output.

In the experiments described the "resistance" and "capacity" effects were produced successively, but clearly the same results would be obtained if the venous reservoir were compressed simultaneously with an increase in the peripheral resistance. The advantage of the "resistance" and "capacity" effects being separated in this manner is that the alterations due to each can also be separated. It was suggested that the insertion, in place of the artificial resistance, of a sponge, enclosed in a rubber membrane and surrounded by a jacket filled with water which was in connection with a calibrated syringe, would enable one to produce the "resistance" and "capacity" effect simultaneously. Pushing home the syringe would reduce the capacity by a known amount, and increase the resistance of the sponge. This was tried, but the results were not satisfactory, because it was difficult to obtain the correct size of sponge mesh to give the required resistance increase for any given capacity diminution.

The results obtained by balancing the pressures and cardiac output bring out several features of the closed circuit preparation which require further consideration. Neglecting any small leakages which may occur, the blood content of the system is constant under all conditions, therefore the total capacity of the heart-lungs and systemic circulation is also constant. Any increase in capacity of one portion of the system must be accompanied by a compensatory diminution in capacity in some other part or in the whole of the remaining portions. In the same way any change which involves a redistribution of blood, so that the blood supply to one part is increased, can only occur at the expense of the blood flow to the rest of the system. It follows that if the coronary blood flow is increased it will only be at the expense of the peripheral flow. Coronary dilatation unaccompanied by any other changes (neglecting the capacity changes due to the coronary vessel dilatation) will cause an increase in the coronary flow and a decrease in the

peripheral flow by the same amount, the cardiac output remaining constant. Increase in coronary flow produced by raising the arterial pressure is complicated by the distension of the arterial system reducing the total output, but when the output* is brought back to its original value by capacity diminution, the pulmonary arterial pressure is found to be unchanged, or only slightly elevated. Tables IV(b), V, and fig. 5.

In the open-circuit heart-lung preparation, Anrep and Bulatao (13) have shown that the total output of the right and left heart increases with every rise in aortic resistance, the increase in output being entirely due to the augmentation of coronary flow; moreover, the main factor which determines the pulmonary arterial pressure is the blood flow, and that the arterial resistance acts only so far as it affects the output of the right ventricle. The fact that in the closed circuit preparation the pulmonary pressure is unaltered, or only shows a slight rise when the peripheral resistance is increased, and when the output is maintained constant, confirms the work of Anrep and Bulatao.

The relative effects of "resistance" and "capacity" alterations upon the mean aortic pressure, when the output of the heart is maintained constant, can be seen from Table IV and fig. 5. In the Table the increase in resistance raised the arterial pressure from 70 to 92 mm. Hg, whereas the capacity decrease necessary to bring back the output of the heart to its initial value raised the pressure from 92 to 106 mm. Hg. That is, 61 per cent. of the arterial pressure rise was due to resistance increase and 39 per cent. to the capacity decrease. In other experiments the percentage of the total rise due to resistance increase was generally a little higher. It is of interest to note that Bayliss and Starling (5) found a total fall of arterial pressure produced by section of the cord in the intact animal equal to 50 mm. Hg, 10 mm. Hg of which was due to diminished peripheral resistance and 40 mm. Hg. to the increased capacity of the system.

Patterson and Starling (14) found that, with constant venous inflow, the greater the peripheral resistance the higher the venous pressure. The same result appears in the closed preparation, for with constant output as measured by the cardiometer, the higher the arterial pressure the higher the venous pressures, but in general the right auricular pressure is only slightly elevated, the absence of a large change being due to the absence of the extra output due to the coronary flow, as shown by Anrep and Bulatao. The slight rise

* It must be remembered that this is measured by the cardiometer, and therefore represents the whole output of the ventricles, i.e., the peripheral outflow, the coronary outflow and the pulmonary arterial outflow.

of pressure in the right auricle when it occurs must be due to "back pressure" effects.

The Pulmonary Arterial Pressure.

When the volume of circulating blood is increased, or when the capacity of the circulation is diminished, measurements of the pulmonary arterial pressure show that it rises with the systemic arterial pressure. Table VI. In three experiments the pulmonary pressure rise was proportionally higher

Table VI.—Dog, 7.5 kilos. Ht. wt., 70 gms. Temp. 35.7° C.

P.A.	M.S.P.	A.R.	$\frac{\text{P.A.}}{\text{M.S.P.}}$	
18	96	55	$\frac{1}{5.3}$	
20	98	55	$\frac{1}{4.9}$	10 c.c. blood injected.
22	102	55	$\frac{1}{4.6}$	Ditto.
24	106	55	$\frac{1}{4.4}$	Ditto.
21	112	70	$\frac{1}{5.3}$	A.R. raised.
18	118	90	$\frac{1}{6.5}$	Ditto.
12	124	110	$\frac{1}{10.3}$	Ditto.

than the systemic pressure rise, so that the pressure ratio P.A.:M.S.P. gradually increased. In a fourth experiment this ratio decreased slightly. The limits in the four experiments were 1:7.2 to 1:5.6, 1:5.3 to 1:4.4, 1:6.7 to 1:4.7, and 1:2.7 to 1:3.0.

With regard to the effect of an increase in arterial resistance upon the pulmonary pressure, it has already been shown that there is invariably a fall; more extended observations given in Table VI demonstrate the fall in the pressure ratio P.A.:M.S.P. Resistance increase and capacity diminution therefore have opposite effects on the pulmonary arterial pressure, and also tend to send the pressure ratio P.A.:M.S.P. in opposite directions. Openchowski (15) gives protocols of experiments in which the pulmonary

pressure varied inversely as the carotid pressure. The explanation would appear to be that "capacity" and "resistance" effects controlled the circulation in the same way as does a rise in peripheral resistance in the closed circuit system. Under these conditions the output of the heart would be diminished.

Effect of an Increase in Peripheral Resistance when Shunted by a Second Resistance.

In the intact animal the systemic circulation is additionally complicated by the presence of numerous shunt circuits, instead of by only one, *i.e.*, the coronary circulation, as in the heart-lung preparation. The effect of shunting the artificial resistance by a second resistance, fig. 1, D', was therefore tried. The results obtained were entirely as expected, and may be stated briefly. On putting up the main artificial resistance, fig. 1, D, the alterations in pressure and output were not so marked as when the shunt circuit was absent. The shunt resistance was only slightly distensible, so that the extra amount of blood passing through it as a result of the raised arterial resistance was insufficient to compensate wholly for the fall of venous pressure and output which usually occurred. On diminishing the resistance of the shunt circuit when the main resistance was up, the venous pressures and output could be made higher than they were before the main resistance was increased; in this case the systemic arterial pressure fell. The compensatory dilatation of shunt circuits in response to a raised arterial resistance was considered by de Jager (4), and Bradford and Dean (16). The work of Anrep and Bulatao is of additional importance in that it shows to what extent shunt circuit dilatation may influence the inflow to the heart. It is clear, therefore, that the effect of the addition of a distensible circuit shunting the main resistance in the closed circuit preparation is to counteract the blood redistribution effect produced by raising the arterial resistance.

Discussion.

In the main, the hæmodynamics of the closed-circuit heart-lung preparation may be condensed into the statements (1) that the effect of a change in peripheral resistance will be determined by two factors, the resistance change itself and the redistribution of blood which it must of necessity produce, and (2) alterations in capacity which will produce a redistribution of blood. Since, within physiological limits the heart can put out as much blood as it receives, the

redistribution of blood in (1) will depend upon capacity changes and the ease with which blood can flow through the main or shunt circuits, and these in turn will be governed by the volume elasticity coefficients of the various parts of the system. Since the resistance, the capacity, and the volume elasticity coefficients of the different parts of the systemic circulation can be varied at will, wide alterations in the distribution of blood can be brought about, which, from the standpoint of being of physiological value, will only be limited by the physical constants of the heart and lungs circulation.

Although the arterial and venous connections were selected to have physical constants as near as possible to those found in the intact animal, it is not considered that the quantitative effects of "resistance" and "capacity" changes which have been found are applicable to the living vascular system. The distension of the arteries due to a peripheral resistance increase may be of minor importance in the intact animal, but it is interesting to note that if the cardiac output is to remain constant or be increased under these conditions there must be a "capacity" diminution or compensatory shunt dilatation.

Summary.

In the closed-circuit heart-lung preparation :—

1. A diminution in capacity of the systemic circulation produces the same effects qualitatively and quantitatively as an increase in the volume of circulating blood, namely, a rise in pressure in both auricles, the aorta and the pulmonary artery, and an increase in the cardiac output.

2. An increase in peripheral resistance causes a fall of pressure in both auricles and in the pulmonary artery, and a diminution in the cardiac output. These changes are due to the raised aortic pressure distending the arterial system, pooling the blood therein, and reducing the inflow to the right heart. The extent of these alterations is dependent upon the "potential capacity increment" possessed by the arterial system.

3. By the simultaneous production of a resistance increase and a capacity diminution, the pressures in the left auricle, right auricle or pulmonary artery, or the output of the heart can be maintained constant.

4. When the systemic resistance and capacity of the systemic circulation are balanced so that the output of the heart remains constant, the pulmonary arterial pressure is unaltered or only slightly elevated. This is in confirmation of Anrep and Bulatao.

5. "Resistance," "capacity," and blood redistribution effects are inter-dependent, their relative effects being determined by the physical constants of the circulation.

The expenses of this research were, in part, defrayed by the Government Grants Committee of the Royal Society.

REFERENCES.

- (1) Weber, 'Ber. ü. d. Verhandl. d. Königl. Sächs. Gesell. d. Wiss.,' Leipzig, p. 196 (1850).
 - (2) Volkmann, 'Hæmodynamik,' Leipzig (1850).
 - (3) Donders, 'Arch. f. Anat. u. Physiol.,' p. 433 (1856).
 - (4) de Jager, 'Journ. of Physiol.,' vol. 7, p. 130 (1886).
 - (5) Bayliss and Starling, *ibid.*, vol. 16, p. 159 (1894).
 - (6) L. Hill and Barnard, *ibid.*, vol. 21, p. 323 (1897).
 - (7) L. Hill, *ibid.*, vol. 28, p. 122 (1902).
 - (8) Connet, 'Amer. Journ. of Physiol.,' vol. 54, p. 96 (1920).
 - (9) Daly, 'Journ. of Physiol.,' vol. 60, p. 103 (1925).
 - (10) Spehl, 'De la répartition du sang circulant dans l'économie,' Bruxelles (1883).
(Quoted by Tigerstedt in 'Die Physiol. des Kreislaufes,' IV, 1923.)
 - (11) Harris, 'Brit. Journ. of Exper. Path.,' vol. 1, No. 3 (1920).
 - (12) Fühner and Starling, 'Journ. of Physiol.,' vol. 47, p. 286 (1913).
 - (13) Anrep and Bulatao, *ibid.*, vol. 60, p. 175 (1925).
 - (14) Patterson and Starling, *ibid.*, vol. 48, p. 357 (1914).
 - (15) Openchowski, 'Arch. f. d. ges. Physiol.,' vol. 27, p. 258 (1882).
 - (16) Bradford and Dean, 'Journ. of Physiol.,' vol. 16, p. 34 (1894).
-

The Heat Liberated by the Beating Heart.

By E. FISCHER (Rockefeller Fellow).

(Communicated by Prof. A. V. Hill, F.R.S.—Received December 21, 1925.)

(From the Department of Physiology and Biochemistry, University College, London.)

Since myothermic methods have been improved several attempts have been made to measure the heat liberated by the beating heart. Two observers, both experienced in the difficulties and limits of myothermic experiments, A. V. Hill (1) and K. Bürker (2), were unable to decide how far their results showed the real change of temperature of the heart, and how far they were the effects of several sources of error. On the other hand, Herlitzka (3), Snyder (4), and Bohnenkamp (5) believed themselves entitled to draw important conclusions from their experimental results. At the suggestion, therefore, of Prof. A. V. Hill, I undertook to investigate the heat production of the heart with the various means available in his laboratory. Since, as will be shown below, my results were not in accordance with those of the last named investigators, I was forced to compare my methods with theirs. According to the most reliable of my observations I believe the warming of the heart (eel or tortoise) for a single beat to be not higher than 0.00012° . For the heart of the rabbit Herlitzka found a rise of 0.004° to 0.012° ; for the terrapin's heart Snyder found 0.00075° to 0.00255° : and for the frog's heart Bohnenkamp found 0.001 to 0.01° .

APPARATUS AND METHODS.

Galvanometer.—The galvanometer employed was designed and constructed by A. C. Downing (6). The "figure of merit" of this instrument (23,000) permitted the use of a high sensitivity, without making the deflection time when critically damped too great. With a sensitivity from 1.2×10^{-9} to 1.3×10^{-10} amps. per mm. the galvanometer had a complete period from 0.6 to 2.5 secs. The four coils, each of resistance 6.6 ohms, could be placed in series, or parallel, or series-parallel, so that the resistance of the galvanometer as a whole could be made to approximate to that of the thermopile, or thermocouple, used in the different experiments. The galvanometer was protected by a double shield of high permeability steel, as described by Hartree and Hill (7). Records were made photographically, as described by them, but with the difference that the

distance of the drum was 1.30 metres and that the light from the lamp was interrupted each half-second by a shutter carried on an electro-magnet actuated by a half-second pendulum.

An arrangement was provided by which a known e.m.f. could be thrown into the galvanometer-thermopile circuit to test the volt-sensitivity of the combination, the degree of damping, or the rapidity of movement. A second, almost similar, arrangement was used to throw such an e.m.f. into the combined circuit as would compensate for the constant temperature difference between the warm and the cold junctions of the thermopile. The e.m.f. so used could be read, and thus it was possible to measure the magnitude of this temperature difference.

Muscle Chamber.—The muscle chamber was similar to that described by Fenn (8). It was deeply sunk under water at any required temperature in a large double-walled silvered vacuum flask. The water inside the flask was vigorously and continuously stirred by bubbling air through it.

Thermal Registration.—The thermopiles used in these experiments were of various kinds. Realising that slipping of the muscle over the junctions would provide the most likely source of error, and supposing that a single couple with one junction inside the muscle of the heart would best avoid this slipping, I first used single couples of silver-nickel-silver, or nickel-constantan-nickel, or iron-constantan-iron. The wires were 0.1 to 0.2 mm. in diameter and the length of each of the three soldered wires was 3 to 4 cms. The middle wire of each couple had at each end a knot, at a distance of 3 to 4 mm. from the junction. By means of a fine needle the soldered wire was pulled through the heart muscle wall until the knot stopped it: at the point where the wire came out of the heart it was fixed by a thread around the wire. The end portions of the couples were soldered to two copper wires coming out of a glass tube through the rubber stopper. By means of its long leads the couple was made elastic enough to allow the heart to move as it required without causing any visible change to occur in the position of the inside junction, which was held inside the muscle at the one end by the wire knot, at the other end by the thread. The cold junction was embedded in a piece of plasticine. The sensitivity given by this arrangement in connection with the galvanometer at the sensitivity mentioned above, was 1.1×10^{-4} to $2.7 \times 10^{-5}^\circ$ for 1 mm. deflection on the drum.

I attempted to increase the temperature sensitivity by using two couples in series, but control experiments, in which an ordinary wire (without junctions), of the same resistance as the couples employed, was pulled through the heart

muscle, showed sometimes that the galvanometer made deflections at each beat. These deflections might be caused, if beginning before the beat, by the action current; if occurring during the contraction, by polarisation phenomena due to the action or the injury current, and connected with the movement of the tissue along the wires or with variation of electrical resistance owing to the change of shape. Control experiments, in which *insulated* wires without junctions were fixed in a similar way inside the heart, never gave any deflection. Thus the deflection obtained when using uninsulated wire passing through the muscle in two different places cannot be caused by electromagnetism, or by currents induced by the action currents, as Bernstein (9) supposed in a similar case.

Control experiments made with an ordinary unsoldered wire, employed in the same way as a single couple, never showed any disturbance of the kind described. Such disturbances are clearly due to contact of a wire at two points with the muscle. I attempted to insulate the junctions so as to be able to employ two junctions or more inside the muscle, but I never succeeded, with the fine wires used, in getting so perfect an insulation that I could be sure of it after the couple had been pulled through the heart. For the same reason I was forced, when using a single couple, to make experiments only in a moist chamber and never in Ringer's solution.

With this method I investigated the hearts of frog, eel and tortoise. The results were not sufficiently satisfactory, and I next attempted to use a thermopile touching the outside of the muscle, on the one hand without preventing movement of the heart, on the other without slipping of the junctions. After many trials I concluded that the shape of the heart and its consistency, in the case, at any rate, of frog and tortoise, make it impossible to fit to it any form of thermopile which does not allow slipping of the junctions. For the longest eel heart I used a thermopile similar to that employed by Herlitzka for the rabbit's heart. The best thermopile employed, which was constructed for me by Mr. A. C. Downing, consisted of a $7/8$ sector of an ebonite ring 1 mm. thick, of which the inner diameter was 4 mm., the outer 13 mm. Its total weight was 0.215 gms. The "warm" junctions were inside the ring, the "cold" junctions outside. The wire employed was 0.12 mm. thick; the 40 couples in it were made by the silver-plating method of Wilson (11). Its resistance was 25 ohms. This thermopile, when fixed on an eel's heart of not too small dimensions, and connected by fine silver wire to the copper leads of the rubber stopper, seemed (so far as was possible to observe by eye) neither to prevent movement of the heart, nor to permit the part of the muscle

touching the thermopile to slip over the junctions. The thermopile was insulated, first by varnishing it with shellac, then by rendering it waterproof before each experiment by a thin layer of solid paraffin. This layer was made by painting the thermopile with a dilute solution of paraffin wax in ether. A thermopile so prepared, employed either in Ringer's solution or in a moist chamber, never gave trouble of any kind. The sensitivity of the whole arrangement, with coils in series in the galvanometer, could be made as high as $1 \text{ mm.} = (7 \times 10^{-6})^\circ$.

In several experiments couples were used as described by Bohnenkamp. Short pieces of constantan and iron wire were soldered together, as shown in fig. 1, and connected by fine silver wires to the copper terminals of the muscle chamber. The heart was placed in the middle so that it was touched by six

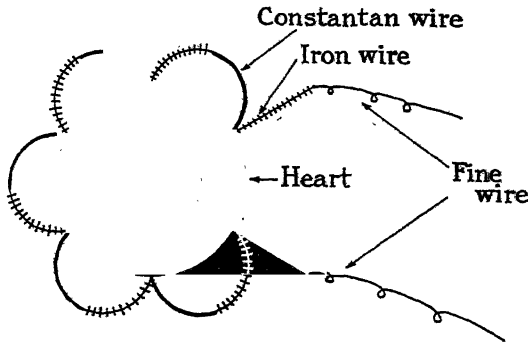


FIG. 1.—Thermopile, similar to that of Bohnenkamp.

junctions. When the heart beat it appeared as though no displacement of any kind took place between the junctions and the muscle where they touched it.

Temperature Sensitivity.—The temperature sensitivity of the single couples was calculated from the theoretical thermo-e.m.f., and of the thermopile with silver-plated couples on the assumption of 20 microvolts per couple per 1° . It was impossible to employ a direct calibration of the thermopile under the conditions of these experiments, owing to lack of uniformity of section of the tissue employed, but from other experiments in which calibration could be carried out about 20 microvolts per couple per degree was found. Comparison experiments with the ring thermopile on a gastrocnemius-sciatic preparation of the frog showed that this number gave results of the same order as found by other methods, namely, $3 \text{ to } 4 \times 10^{-3}^\circ$ rise of temperature for a single maximal twitch.

Heat-Loss.—Since the wires were thin and the distance between cold and warm

junctions was great, particularly when using a single couple, it was to be expected that "settling down" would be slow after a rise of temperature had taken place. Using a single couple on a gastrocnemius muscle the galvanometer made, after a single induction shock to the nerve, a quick deflection of a certain size, remained in its new position for several seconds, and began then still further to deflect, in consequence of the recovery heat production. Sudden warming of the gastrocnemius gave a rapid deflection without any appreciable return of the galvanometer during the first few minutes.

The "settling down" of the ring-thermopile took several minutes to complete, even in experiments in Ringer's solution. In the moist chamber half of the temperature rise recorded was lost in 14 seconds, in Ringer's solution in 8 seconds. Using a gastrocnemius stimulated through its nerve, instead of a heart, the ring thermopile employed in Ringer's solution gave a deflection only 16 to 17 per cent. less than in a moist chamber.

Mechanical Recording.—As is known, the best method of preventing any slipping over the junctions is to let the muscle make only isometric contractions. For the heart this implies that it must beat always on its diastolic filling without ejection. No heart will do this for any considerable time, as is necessary for preliminary equalization of temperature in myothermic work. It is impossible to get even an approximately isometric contraction of a heart by fixing one point of it to an isometric lever. The heart, therefore, was connected only to an ordinary heart lever: one end of this lever was bent down and insulated and ended in a platinum point dipping in a small vessel filled with 95 per cent. alcohol with a trace of NaCl dissolved in it. Opposite the platinum point of the lever was a second platinum point brought insulated into the vessel. As the heart worked the distance between the two platinum points changed, and consequently the electrical resistance between them. By means of a Wheatstone bridge this change of electrical resistance was indicated on a Kipp galvanometer. By means of a lamp and mirror the deflections of this galvanometer were marked on the same drum as those of the Downing galvanometer. This arrangement showed practically no polarisation and worked with very small delay, the galvanometer curve being very nearly the same as the curve of movement of the heart lever itself.

Work by Heart.—In a few experiments the heart was fitted with a cannula through the aorta and valve, so that as it beat it moved the liquid in the cannula up and down, and so did work. This arrangement, however, appeared only to introduce a new source of error without appreciably bettering the conditions,

and I returned to the heart working without filling. In the experiments with the eel's heart in Ringer's solution I tied into the vein, as well as into the aorta, a small cannula: the one in the vein was intended only to keep the vein open so that the Ringer's solution could reach the auricle: the aorta cannula ended 30 mm. above the surface of the Ringer's solution. In this way the heart did a certain amount of external work.

Temperature Equalisation.—After all preparations had been made, and the air had started bubbling through the water of the vacuum flask, it was necessary to wait for at least an hour before making a record. Even so, in all my experiments, there was a considerable difference of temperature between the "cold" and the "warm" junctions. In agreement with Bürker (10) I found in the moist chamber that the "warm" junctions were nearly always colder than the "cold" ones. In Ringer's solution the difference was much smaller, and now in the other direction, the "warm" junctions being hotter than the "cold" ones. No attempt was made to eliminate this difference by covering the cold junctions with moist tissues, since in this way one cannot avoid the difference of temperature between different points in the chamber itself: one can only equalise the temperature of the cold and the warm junctions. I preferred, therefore, to compensate the temperature difference with an electrical current, as described above. This temperature difference indicated, to a certain degree, the magnitude of the differences likely to occur between different parts of the chamber, and therefore also of the heart. I supposed that, in general, the smaller the difference between the "cold" and the "warm" junctions, in experiments of this kind, the smaller would be the errors due to slipping over the junctions.

After each experiment the direction of the deflections corresponding to a rise of temperature of the muscle was carefully tested.

RESULTS.

Experiments with Single Couples.—The first experiments were made with a frog's heart, using single couples. In agreement with other investigators they showed a process with two phases. The first phase begins with systole and ends with, or shortly after, the beginning of diastole. Now begins the second phase in the opposite direction, outlasting diastole for a short time. The movement of the first deflection is generally quicker than of the second. In order to avoid too rapid a beat the experiment was carried out, either at a low temperature (12° C.) or by using the ventricle only, beating in its own rhythm. In many experiments, especially with eel hearts, single beats were

produced by interrupting a vagus stimulation. Some such device is necessary in order to cause the heart to beat so slowly that the galvanometer may follow its temperature changes with a certain accuracy.

The results of the experiments with single couples were remarkable for two facts, (a) the great variation of the temperature change involved, and (b) the sign (\pm) of the first temperature deflection. With regard to (a) the magnitude of the temperature change varied in different experiments in an extraordinary manner, viz., from 0.00014° to 0.0031° . The average of a number of experiments was 0.00094° . The deflections became smaller when the heart began to beat more feebly. Sometimes an increase of the deflection was noted after a long vagus stimulation, but at the same time the amplitude of the heart beat increased also. Without any strict proof I formed the impression that, in a given heart, the size of the galvanometer deflection was proportional to the amplitude of the movements. With regard to (b), the direction of the initial heat change was, in different hearts, not always the same. As these were my earlier experiments I neglected sometimes to test the direction of the deflections. In 25 per cent., however, of the observations the first deflection corresponded to a *warming* of the heart muscle, and in 50 per cent. of them to a *cooling*.

It is impossible to believe that variations, in the ratio of 22 to 1, of the initial heat deflection can have a physiological origin. The same thing, however, has been observed by others. Herlitzka found in his experiments that one rabbit's heart might give a temperature change of 0.012° C., while another heart gave apparently nothing. (His temperature sensitivity was 1 mm. = 0.006° C., and his string galvanometer curve was readable to 0.1 mm.) Bohnenkamp also found differences in the ratio of 10 to 1. Still less reasonable is it to suppose that the physiological process may in one case be endothermic and in the other exothermic. Moreover, the fact that for each beat the *total* thermal effect was always zero was suspicious. With a *regularly* beating heart we might expect, as A. V. Hill (1) pointed out, regular oscillations of the galvanometer, since the heart would go on warming until it reached a steady state, in which heat-loss in diastole was balanced by heat-production in systole. In experiments, however, in which a *single* beat occurred, after stopping the heart for a long time, the total effect was still zero and the return to zero was much quicker than would be possible were it due simply to loss of heat.

Snyder, as well as Bohnenkamp, concluded that the second phase could not be due to loss of heat; the former imagined a second chemical process to occur with negative heat production, the latter a physical endothermic process

(charging of colloids). In the present experiments, however, it seems much more probable that, if the direction also of the initial heat may vary, then all these remarkable results should be attributed to some technical error.

I attempted, therefore, to repeat the experiments with a heart much more solid and compact than the spongy frog's heart. The eel's heart seemed well fitted for this purpose, particularly because it lives much longer in the moist chamber than does that of the frog. The results, however, were no better. I obtained values varying from 0.0002 to 0.004° with an average of 0.00168 . In one-third of these experiments the initial deflection was positive, in two-thirds negative.

The experiments were repeated therefore with tortoise hearts. At first I was unable to obtain tortoises with hearts in sufficiently good condition to function long enough in the moist chamber. Only shortly before the completion of the experiments recorded here did I get some tortoises fit for my purpose, so that there are only a few experiments to record.

As I imagined that the chief error was caused by movement of the heart, bringing the "warm" junction in one case more to the inside, in the other more to the outside, I attempted in several experiments with the thicker tortoise heart to place the junction (*a*) more to the outside (1 mm. distance); (*b*) nearer to the inside, and (*c*) as nearly as possible in the middle of the muscle. The position of the junction was determined after each experiment, by section of the heart.

No.	Date.	Temperature change, $^\circ\text{C}$.	Direction of first deflection.	Remarks.
1	10.12.25	0.0017	—	Junction near the outer surface.
2	10.12.25	0.00064	—	Junction touching the inner surface.
3	11.12.65	0.00012	+	Junction in the middle.
4	14.12.25	0.00010	+	Junction in the middle.
5	16.12.65	<0.00010	?	Junction in the middle.
6	17.12.25	0.0015	—	Junction touching the inner surface.

It must be admitted that in experiments 3, 4 and 5 the duration of second phase was so long that it could not be said with certainty that this phase is not due to temperature equalisation.

No definite conclusion could be drawn from these results. On the presumption that different hearts have a heat development of the same order of magnitude, one can only conclude that this does not result in a rise of temperature of more than 0.0001° for a single beat. In favour of this conclusion is the fact that in frog and eel hearts the deflections, even when as small as 0.0002° ,

may be in either direction (+ or -), which could scarcely be the case were there a consistent positive heat production of an order of size much greater than that quantity.

Experiments with Ring Thermopile.—Experiments carried out with the ring thermopile in a moist chamber gave no better results, although the position of the thermopile (as far as one could judge by eye) was an ideal one. The records had two phases as already described. The temperature deflections varied from 0.00008 to 0.003°, with an average of 0.00134°. In half the experiments the first deflection was positive, in half negative. These experiments were continued sometimes for five or six hours; in two of them I observed a change of direction of the galvanometer deflection to occur without any external cause, while in a third a change of direction happened after a short and gentle pull by the hand on the lever. Since these results were as unsatisfactory as those with a single couple I attempted to repeat them in Ringer's solution. As mentioned above, the temperature sensitivity of the apparatus is diminished only 17 per cent. by using Ringer's solution around the muscle and thermopile. The first difficulty encountered was to get a Ringer's solution fitted for the eel's heart. It was found, however, by experiment that an oxygenated solution of 0.7 per cent. NaCl, 0.015 per cent. NaHCO₃, 0.01 per cent. KCl, 0.03 per cent. CaCl₂, and 0.1 per cent. glucose, in glass-distilled water, enabled one to keep the heart beating sometimes as long as 18 hours.

These experiments in Ringer's solution *all showed a positive initial deflection*, while the following negative one was so slow that it was impossible to decide that the second phase was not due merely to loss of heat. On the average, the warming effect was only 0.000071°, the greatest being 0.000112°, the smallest 0.000047°. These experiments make it still more probable that the real thermal effect in the single heart beat is of the order of 0.0001° C.

Some experiments were carried out in another way, the deflection being observed first in Ringer's solution and then after removal of the solution from the chamber. The following is a typical experiment.

No.	Time after beginning of experiment.	Deflection, °C.	Direction of first deflection.	Difference between "cold" and "warm" Junction, °C.
	hr. mins.			
1	1 20	0.000061	+	- 0.0025
	1 21	Removal of Ringer's solution.		
2	1 40	0.000084	+	+ 0.0058
3	2 10	0.00015	+	+ 0.00325
4	2 40	0.00014	+	+ 0.00275

Such experiments showed that after the removal of the Ringer's solution the deflection increases with the time, but never reaches higher values than 0.0002°C . In some experiments the direction of the deflection changed immediately after removal of the solution.

In another type of experiment, a record was first made in the moist chamber, then the chamber was filled with Ringer's solution and after some time another record was taken.

No.	Time after beginning of experiment.		Deflection, $^{\circ}\text{C}$.	Direction of first deflection.	Difference between "cold" and "warm" junctions, $^{\circ}\text{C}$.
	hr.	mins.			
1	1	0	0.0013	+	- 0.02
	1	1	Ringer solution brought into the chamber.		
2	2	0	0.000047	+	- 0.0059

Such experiments made it evident that the irregular results obtained in experiments with the moist chamber were due to unsatisfactory conditions there. The greatest care was taken that the chamber should be saturated with moisture. In experiments where a moist chamber at room temperature was placed in the Dewar flask filled with water at a low temperature, great differences between the "hot" and the "cold" junctions existed (0.01° to 0.04°) although at the end of the experiment the tube of the moist chamber had been completely covered with moisture on its inside. As many other investigators have had the same difficulty it seems necessary to suppose that in the moist chamber evaporation is taking place in some spots, condensation at others. By filling the chamber at first completely with Ringer's solution and then blowing it out the conditions can be improved, as Hartree and Hill have pointed out. It may be that this improvement is due to better equalisation of temperature, since heat conduction and convection in the Ringer's solution is much quicker than in air. The equalisation, however, is never complete even in Ringer's solution.

Temperature Differences in the Muscle.—It is obvious that the cause of disturbances due to slipping over the junctions must be differences of temperature along the muscle itself. When a ring thermopile is employed it is probable that the covered portion of the muscle has another temperature than the uncovered. Some part of the disturbance, moreover, may be due to the fact that the firmness of contact between a given area of the muscle and the thermopile changes while the heart passes through its cycle. It was interesting, therefore, to enquire whether such temperature differences do actually exist

in the muscle. For this purpose I used, not a beating heart but a thick gastrocnemius, bringing one junction of a single couple into the middle of the muscle and the other junction 1 to 2 mm. below the surface. Observations were made for more than 48 hours and showed that there was always a temperature difference of some hundredths of a degree. Since this experiment, however, might be disturbed by a difference of electrical potential causing a current in the wire, or possibly by rigor mortis not beginning in all fibres at the same moment, it was necessary to measure the magnitude of such differences as may exist in non-living non-conducting substances in the same moist chamber. For this purpose a piece of plasticine, some centimetres long and 3 to 4 mm.

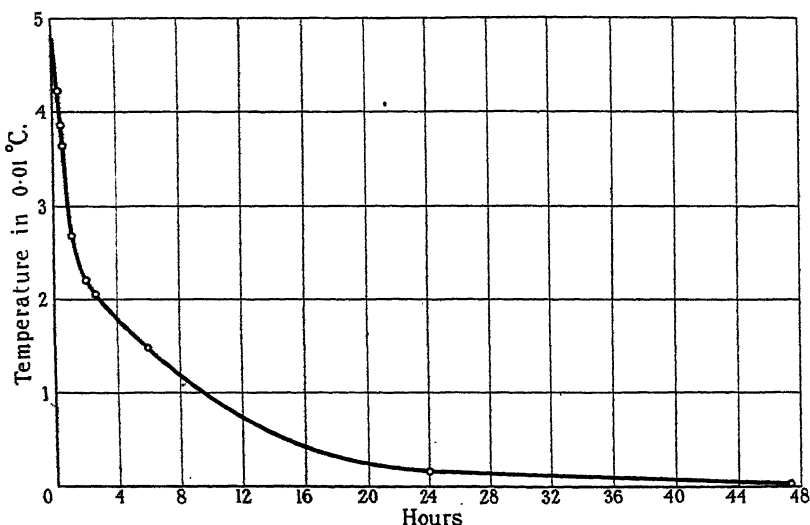


FIG. 2.—Temperature differences at two points 10 mm. apart, in a lump of plasticine suspended in a muscle chamber of the type employed in the present investigation. Vertically, hundredths of a degree; horizontally, time in hours.

thick, was hung vertically in the chamber, the two junctions being placed in the plasticine 10 mm. apart. Fig. 2 shows that the settling down between the two spots takes a very long time. The difference soon (1 hour) reaches a magnitude of 0.03° C., and then falls very slowly till, after 48 hours, it reaches zero.

To compare my methods with those of others I made the same model experiment under the conditions which Snyder used for his heart observations. Snyder employed a muscle chamber consisting of a Dewar flask with a rubber stopper. To produce a low temperature ice was placed in the bottom of the

flask. The results of this model experiment are shown in fig. 3. The difference is 100 times as great as in my arrangement. This indeed is not to be wondered at when one considers a temperature fall of 20 degrees or so between the top of the rubber stopper and the bottom of the flask.

In my experiments the temperature fall between the top and the bottom of the muscle chamber was very small, since it was kept in the moving water of the Dewar flask. If the air was bubbling well the top of the water in the flask was only seven to eight-thousandths of a degree warmer than that at the bottom. In the absence of bubbling, however, the top was soon 0.17 degree cooler than the bottom.

Since the outer temperature of the muscle chamber, as employed in my experiments, was so nearly the same at different points, the relatively high differences

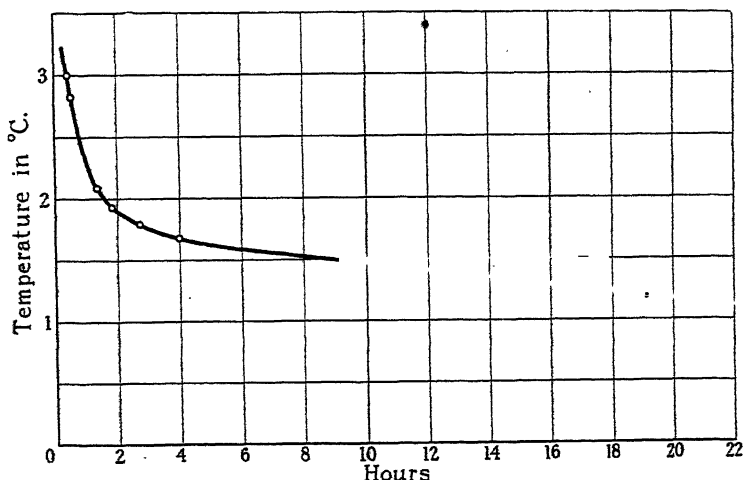


FIG. 3.—Temperature differences at two points 10 mm. apart, in a lump of plasticine suspended in a muscle chamber similar to that employed by Snyder, with ice at the bottom. Vertically, degrees; horizontally, time in hours.

in the inside cannot be attributed to inequalities of the outside temperature: they must be caused either by evaporation, or by differences in the slow rate of "settling down." In any case the existence of such temperature differences makes it clear why slipping over the junctions, even of invisible dimensions, may cause disturbances relatively so great.

Even in the method employed by Bohnenkamp there must be some change in the position of the junctions, though due perhaps only to an alteration of the pressure of the junctions on the tissue during systole. Experiments carried

out with his method gave me also deflections of 0.001 to 0.01°C . Used with a gastrocnemius his thermopile gave not only a warming on contraction but also a cooling on relaxation. The same effect has been found by Bohnenkamp himself, but is never obtained with ordinary well-proved methods.

Discussion.—The results of my experiments are therefore unfortunately mostly of a negative kind. It would seem probable that the thermal change in the cold-blooded heart is only of the order of 0.0001°C . per beat. Further experiments must be carried out in order to be certain that even this number is not too large. The experimental difficulties are great, and it would seem certain that the preponderant effect in all observations recorded so far is one due purely to physical errors and to imperfections of technique. It is natural that the heat production of the heart should be so small, since the intrinsic force of heart muscle is much less than that of skeletal muscle. Since the results are so uncertain it would seem inadvisable to make any further theoretical discussion of them.

Summary.

1. An attempt has been made, by various methods, to determine the heat produced during the beat of a cold-blooded animal's heart.

2. Under ordinary conditions a thermojunction sewn into the heart muscle, or a thermopile resting against its surface, may show large and easily recorded effects: three facts, however, show that these are due, mainly or entirely, to imperfections of technique: (1) the extreme variability of the initial change of temperature during systole, in the absence of any corresponding variation in the heart beat itself; (2) the absence of any constancy in the direction of this initial heat change, which is as often negative as positive; and (3) the fact that the thermal change in systole is almost exactly balanced by an equal and opposite change in diastole. It is concluded that these obvious effects are due mainly, or entirely, to movement of the thermojunctions over, or through, the tissue.

3. Such movements could produce apparent thermal effects only if there were differences of temperature along the surface, or inside the substance, of the muscle. Direct experiments with pairs of thermojunctions in different parts (*a*) of a heart, (*b*) of a gastrocnemius muscle and (*c*) of a lump of plasticine, suspended in a moist chamber, have shown that temperature differences do exist, sufficient to explain the observations, even after considerable intervals have been allowed for temperature equalisation, and even when great care has been taken to ensure equality of the external temperature.

4. These temperature differences can be largely avoided by suspending the tissue and thermopile in Ringer's solution : without affecting the magnitude of the heart beat, the introduction of Ringer's solution into the chamber may largely abolish, or even change the direction of, the thermal deflections previously observed.

5. The heart muscle is so ill-adapted to myothermic experiments, and the heat production during a heart beat seems to be so small, that it would appear impossible, by any method hitherto described, to measure the true rise of temperature with any accuracy, still less to draw any conclusions from the records obtained.

6. In a general way it is concluded that the rise of temperature in a cold-blooded heart, contracting isotonically against a small load, is of the order of 0.0001°C .

It is a pleasure here to express my indebtedness to Prof. A. V. Hill for his advice and assistance throughout this work, and to Mr. A. C. Downing for his help with thermopile and galvanometer. As the holder of a Fellowship of the Rockefeller Foundation I wish to express my thanks also to that body for making this work possible.

REFERENCES.

- (1) A. V. Hill, 'Ergeb. der Physiol.,' vol. 15, p. 341 (1916).
- (2) Bürker and Bruns, v. Weizsäcker, 'Handb. d. Biolog. Arbeitsmethoden,' vol. 5, Abt. 1, Lief. 63, p. 160 (1922).
- (3) Herlitzka, 'Arch. di Fisiol.,' vol. 10, p. 501 (1912); 'Pfüger's Arch.,' vol. 161, p. 397 (1915).
- (4) Snyder, 'Amer. Journ. Physiol.,' vol. 44, p. 421 (1917); vol. 47, p. 156 (1918); vol. 59, p. 254 (1922); vol. 63, p. 581 (1923).
- (5) Bohnenkamp, '9 Tag. d. Deutsch. Physiol. Gesell., Rostock' (1925); 'Ber. ü. d. ges Physiol.,' vol. 32, p. 687 (1925).
- (6) Downing, 'Journ. Scient. Instruments' (May, 1925).
- (7) Hartree and A. V. Hill, 'Journ. Physiol.,' vol. 54, p. 85 (1920).
- (8) Fenn, 'Journ. Physiol.,' vol. 58, p. 175 (1923).
- (9) Bernstein, 'Pfüger's Arch.,' vol. 161, p. 595 (1915).
- (10) Bürker, 'Pfüger's Arch.,' vol. 109, p. 217 (1905).
- (11) Wilson, W. H., 'Proc. Physical Soc., London,' vol. 32, p. 326 (1920).

Experimental Studies on the Differentiation of Embryonic Tissues growing in vivo and in vitro.—I. The Development of the Undifferentiated Limb-Bud (a) when Subcutaneously Grafted into the Post-Embryonic Chick and (b) when Cultivated in vitro.

By T. S. P. STRANGEWAYS, Lecturer in Special Pathology, University of Cambridge, and HONOR B. FELL, Ph.D., Beit Memorial Research Fellow.

(Communicated by Sir William Hardy, F.R.S.—Received October 3, 1925.)

(From the Laboratories of the Research Hospital, Cambridge.)

[PLATES 5-13.]

Introduction.

One method by which the problem of the differentiation of animal tissues may be approached is by studying the behaviour of simple embryonic tissues when growing in an abnormal environment, such as that produced by grafting into atypical situations *in vivo* or by cultivation *in vitro*. It is along these lines that the investigations of the present writers are being conducted. The work so far completed, the results of which are recorded in the present communication, consists of a study of the development of the undifferentiated, embryonic limb-bud of the fowl when grafted subcutaneously into a post-embryonic chick and when cultivated *in vitro*. A preliminary investigation of the histogenesis of cartilage and bone in the limbs of the embryonic fowl was carried out by one of the writers (Fell, 1925) in order to provide normal standards with which to compare the experimental material.

Rous (1910, 1911), Fichera (1909) and many others have successfully grafted foetal and embryonic tissues into young and adult animals, usually in connection with the study of tumour growth; a bibliography and summary of the earlier work is given in Fichera's paper. Almost all the work on the development of grafts of the undifferentiated limb-buds has been carried out on the embryonic Amphibia by Braus, Harrison (1907, 1918, 1921), Detweiler (1918, 1925), Nicholas (1924) and others. Spurling (1923) describes a case of accidental but successful autotransplantation of the posterior limb-bud in a fowl embryo. Murray and Huxley (1925) record two experiments in which part of the limb-bud of a four-days' embryo was successfully grafted on to the chorioallantoic membranes; in one case "a highly differentiated and very easily recognizable femur" showing early ossification was found after 5 days' growth.

A fragment of vertebrate embryonic tissue when cultivated *in vitro* may show two types of growth: (1) an outwandering of the cells into the culture medium where they continue to multiply by mitotic division; this form of growth has been termed "uncontrolled" (Thomson, 1914; Fischer, 1922), and "histiotypic" or "cytotypic" (Maximow, 1925). (2) The growth by mitotic division of the whole fragment, which behaves almost like an independent organism and may undergo a certain amount of progressive differentiation. Thomson (1914) calls this type of growth "controlled" but Maximow (1925) prefers the term "organotypic."

The latter type of growth has at present received comparatively little investigation. It was first observed in the Amphibia. Thomson (1914) noted that portions of the embryonic chick, and even whole embryos, increased in size during cultivation *in vitro*, whilst an outgrowth of branching cells occurred only from cut surfaces. Fischer (1922) found that fragments of the small intestine of fowl embryos became completely invested by cylindrical epithelium and remained alive for a month. Chlopin (1922) describes progressive differentiation *in vitro* of various mammalian tissues including fragments of partially differentiated limb-buds. Maximow (1925) studied cultures of portions of early rabbit embryos and noted that the explants underwent organotypic growth, and for a time showed progressive evolution of the various primordia. In Part II of the present communication the writers have shown that the undifferentiated limb-bud of the fowl, when cultivated in a relatively large volume of medium, shows organotypic growth in a marked manner, and at the same time undergoes considerable differentiation along comparatively normal lines.

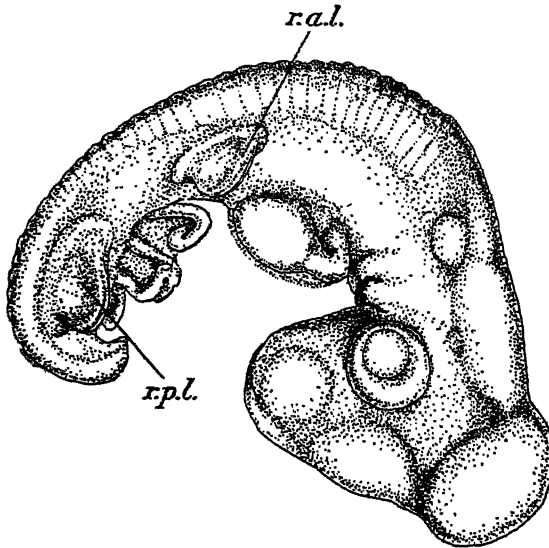
The expenses of the investigations recorded in this paper were defrayed by the Medical Research Council, and the writers have pleasure in expressing their thanks for this assistance.

PART I.—THE DEVELOPMENT OF THE LIMB-BUD WHEN GRAFTED INTO THE POST-EMBRYONIC CHICK.

1. *Technique.*

(1) *Material.*—The chicks and embryos employed in this part of the investigation were from a strain of pure bred White Leghorns. The embryos from which the undifferentiated limb-buds were taken were of 81 hours' incubation, and the chicks into which the buds were grafted were 11 days' old. Twenty-seven chicks were operated upon and twenty-five successful grafts were obtained.

(2) *The Dissection*.—The shell and membranes covering the large end of the egg were removed, and the embryo was lifted out and placed in a drop of sterile Ringers' solution. The right posterior limb-bud (text-fig. *r.p.l.*) was amputated whilst the left posterior limb-bud was fixed in Zenker's Fluid to serve as a control.



TEXT-FIGURE.—Drawing of 80 hrs. embryo showing the limb-buds (*r.a.l.* : right anterior bud, *r.p.l.* : right posterior bud).

(3) *The Operation*.—The limb-bud was inoculated in the under-surface of the wing beneath the skin covering either the prepatagium or the muscles surrounding the radius and ulna.

(4) *Histological Methods*.—The chicks were killed at short intervals and the grafts removed and fixed in Zenker's Fluid. Complete serial sections of each graft and the surrounding host tissues were cut. Sections were stained by iron hæmatoxylin alone, iron hæmatoxylin and Van Gieson's Stain, Mallory's Stain, safranin and picro-indigo-carmin, methylene blue and alcoholic eosin, and hæmatoxylin and alcoholic eosin. Histological preparations of the control limb-buds were made by the same methods.

2. Growth and Differentiation of Grafts.

(1) *Structure of Limb-Bud at Time of Inoculation*.—The posterior limb-bud of an 80 hours' embryo, which varies somewhat in its degree of development in

* The actual inoculation was performed by T. S. P. S.

different individuals, is a flattened structure, semicircular or oblong in shape. In most cases the bud is completely undifferentiated (fig. 1) and consists of a mass of stellate mesenchyme cells covered by a simple ectoderm composed of a basal layer of columnar cells and a superficial layer of flattened cells. Along the distal margin of the bud the ectoderm is thickened to form a small ridge (*r*). In Nos. 19 to 27 the controls show a slightly more advanced stage of development, the mesenchyme having condensed to form an elongated axial mass of precartilage whilst the sites of future muscles are just distinguishable. In no instance has early cartilage appeared. (See Plate 5, etc.)

(2) *Macroscopic Observations on living Grafts.*—As a rule outward signs of growth are not visible until the beginning of the third day, when a small nodule appears at the site of inoculation. This increases in size, growth being very rapid between the fourth and seventh days. In some cases the graft continues to enlarge until about the thirteenth day, but more often growth ceases between the eighth and ninth days. The ultimate diameter of the graft after 13-14 days' growth varies from 4-8 mm.

(3) *The Histological Development of the Grafts.*—The youngest grafts fixed were of 16 hours' growth. Histological preparations show the inoculated limb-bud (fig. 2*g*) lying loosely in the subcutaneous tissue of the host, which is considerably inflamed. The graft itself is composed of a dense mass of undifferentiated cells (fig. 3) more or less polyhedral in outline and containing a large spherical nucleus with plasmasomes; no cartilage is present, but the dense aggregation of the cells probably corresponds to "precartilage." The cells are in active multiplication, mitotic figures being everywhere abundant. Grafts of this age are either not vascularised or merely show a few capillaries beginning to penetrate the surface. Granulocytes together with a certain proportion of other wandering cells are especially numerous around the graft (fig. 2), which may be enclosed by a dense mass of these elements. Always a few and sometimes large numbers of these cells migrate into the limb-bud itself (fig. 3*g*), but apparently have little or no effect upon its growth. The ectoderm of the limb-bud is usually somewhat damaged by the inoculation, and in places may be completely rubbed away. It would seem that a large proportion, if not the greater part, of this epithelium degenerates: the cells shrink and the epithelium breaks up into small areas; the cells composing these fragments begin to liquefy, the intercellular boundaries become obscure and finally disappear, and each epithelial fragment gives rise to a single multinucleated mass of cytoplasm; the nuclei also degenerate and ultimately appear as small crumpled vesicles. In this way numerous multinucleated "giant-cells," of various sizes

and of ectodermal origin, are formed at different points at or near the surface of the graft. The areas of ectoderm which do not degenerate in this manner proliferate with extreme rapidity as indicated by the large number of mitotic figures present; partly by proliferation and partly by an actual rearrangement or condensation of the cells each gives rise either to a solid sphere, or to a cord of cells which penetrates the underlying mesenchyme (fig. 4 *e.c.*) or grows parallel with the surface of the graft. The inner layers of cells in these structures are almost always more flattened than those at the periphery and represent the flattened, superficial cells of the epidermis.

After 40 hours' growth nodules of cartilage appear in the graft; they are usually from three to five in number and occupy the bulk of the graft. The cartilage is mostly of the early "epithelioid" type (fig. 5), *i.e.*, it consists of a mass of polyhedral cells separated from one another by narrow, deeply staining walls of matrix; sometimes development is further advanced and a greater quantity of matrix is present. There is no perichondrium, the cartilage fading gradually into the surrounding mesenchyme. The formation of new solid epithelial bodies is now practically at an end, although an occasional tubule in early development may still be observed; many of these structures have undergone degeneration, giving rise to one or several large multinucleated "giant cells." Most of those which persist are growing actively, and in the majority a lumen is beginning to appear in the form of several small, spherical or oval cavities between the inner layers of flattened cells. Numerous granular polymorph cells wander into the epithelium and pass into the small cavities, where they break down. In cases where the epidermis of the host has healed the epithelium covering the surface of the scar often forms one or more cords which penetrate into the underlying graft. Vascularization begins at this stage. A plexus of capillaries and small vessels is seen growing into the inoculated tissue, but as yet has not penetrated beyond the outer regions of the graft.

By the third day the cartilage is well developed, the inner cells of the nodules being larger and separated by broader walls of matrix, whilst the periphery of the masses of cartilage is gradually becoming more distinct. The blood vessels are more numerous and now extend well into the interior of the graft; a few vessels show regression. The small cavities which appear in the solid epithelial islets after 20 hours' growth enlarge and fuse with one another, so that by the third day most of the epithelial bodies have become completely hollow. In some cases this canaliculization of the tubules may be deferred until the fifth or sixth day (fig. 6, *ep.t.*). All the tissues of the graft show very active cell division.

After four days' growth almost all the cartilage cells have enlarged to about one and a half times their previous size. A few of the cartilaginous nodules have now become invested by a definite perichondrium composed of elongated, flattened cells among which run a few white fibres. In most of the nodules, however, a perichondrium is either not developed at all or is only formed over certain areas of the surface; it is found that this imperfect development of the perichondrium usually persists throughout the life of the graft. Lymphocytes and other non-granular wandering cells are in places migrating from the capillaries and invading the graft tissue. These cells show a tendency to aggregate around epithelial tubules, and many of them wander in among the epithelial cells; this occurs even in cases where the epithelium, to judge by the large numbers of mitotic figures present, is still in a healthy condition.

As development proceeds, the amount of lymphoid tissue present in the graft usually increases enormously, and by the sixth or seventh day the cells of the original mesenchymatous stroma can hardly be distinguished among the mass of immigrated cells. Not only are fresh supplies of lymphoid elements being brought into the graft by the blood stream, but those already present multiply vigorously by mitosis. This rapid infiltration of the implant probably accounts for the strikingly quick increase in size which is often noted in the living graft between the fifth and seventh days of growth.

The epithelial structures continue to grow actively, and by the sixth day have given rise to long, winding clefts and tubules (figs. 6 and 7, *ep.t.*), a single one of which may extend from end to end of the graft. In some of the nodules of cartilage two distinct histological regions are seen. One of these regions is composed of cartilage cells which have become greatly enlarged and separated from one another by wide partitions of matrix; many of these elements are degenerating: the nucleus has become pycnotic and the cell has shrunk into a small, deeply-staining mass. The other region, which consists of smaller cells set more closely together, shows little or no degeneration and usually completely or partially encloses the areas of hypertrophied elements. Similar areas of hypertrophied cells occur in the normal embryonic leg and represent future centres of ossification.

By the seventh day of growth early signs of degeneration are usually beginning to appear. The epithelial tubules show progressively less mitosis, whilst the inner, flattened cells become keratinized and sloughed off into the cavity. Where areas of enlarged chondroblasts occur in the cartilage, the zone of cells separating such areas from the small-celled cartilage, usually begin to lose their intermediate character and become flattened, as if compressed by the growth

of the two adjacent regions. In the normal embryonic limb, a similar belt of flattened cells is formed between the epiphysis and diaphysis, or between the areas of hypertrophied cells and the surrounding small-celled cartilage in the fused tarsal elements. In many of the cartilage nodules of a seven days' old graft the periphery is necrotic: the matrix is disintegrating and is being invaded by the cells of the surrounding stroma, and by numerous oxyphil granulocytes, whilst the liberated chondroblasts degenerate. In places where the cartilage is covered with a fibrous perichondrium this superficial necrosis is not observed. Mitosis, though still present, is diminishing and cell degeneration is becoming more abundant.

Between the tenth and the fifteenth days of growth bone usually appears. Periosteal ossification in the growth always takes place in association with a more or less cone-shaped process of cartilage composed of hypertrophied cells. Although the shape of the cartilage nodules is extremely irregular the three regions of cells characteristic of cartilage undergoing ossification are now very distinct (fig. 8). The small-celled or epiphysal region (*ep.r.*) is sharply marked off from the adjacent zone of flattened cells (*z.f.c.*). It is often more extensive than the projection containing enlarged chondroblasts (*z.h.c.*) which corresponds histologically with the diaphysis. As in the normal embryonic limb the epiphysal type of cartilage becomes honeycombed by a system of canals enclosing blood vessels and a small quantity of connective tissue. When ossification begins, a thin layer of bone is formed over the surface of the region of hypertrophied cells. Where the ossification centre is covered by a perichondrium of elongated, flattened cells, the cells of the inner layers of the perichondrium assume the character of osteoblasts and become included in the lamina of bone as bone corpuscles; the material at the writers' disposal has not permitted them to follow the further progress of ossification in such cases. When no definite perichondrium is present a thin sheath of bone is deposited around the cartilage in precisely the same way, but the origin of the osteoblasts is very difficult to trace owing to the varied character and dense arrangement of the surrounding stromal cells. It would seem, however, that these elements are derived from the cells of the original mesenchymatous stroma which are scattered among the immigrated lymphoid tissue.

In most of the older limb-bud grafts a number of white fibrous membranes are present which run around and between the various nodules of cartilage, in places uniting with one another, in other places fading into the cellular stroma (fig. 9, *f.m.*). These fibrous lamellæ are often continuous in places with perichondrial tissue at the surface of the cartilage, but for most of their area they

are separated from the nodules by a wide space occupied by stromal cells, and their course bears little or no relation to the contour of the nodules. In view of these facts they can hardly be regarded as representing true perichondrium. As periosteal ossification advances, it is found, however, that these membranes serve as a limit to bone formation in the same way as does the fibrous layer of the periosteum in the normal limb. The original bony sheath becomes considerably thickened, and numerous long irregular trabeculae running out into the surrounding tissue in all directions are formed in association with the primary layer. As soon as the trabeculae come in contact with one of these fibrous membranes further growth in that direction ceases and irregular lamellae of bone are laid down immediately within, and running parallel with, the sheet of fibrous tissue. A similar phenomenon occurs in the normal development of the limbs in the fowl (Fell, 1925, p. 430).

Soon after the formation of the primary layer of periosteal bone the outer region of the cartilage may become calcified, and in some cases is invaded by blood vessels, connective tissue and osteoblasts in the usual way. The earliest stages in the invasion of the cartilage are not present in any of our specimens, but one nodule of cartilage (in graft 4, fig. 9) shows this process at an advanced stage. At one end, and along most of one side of this nodule, are strips of cartilage of the epiphysial type (*ep.r.*), which are separated from the region of hypertrophied cells (*z.h.c.*) which forms the main bulk of the fragment by narrow zones of flattened cells (*z.f.c.*). The periosteal bone (*p.b.*) is very well developed around the region of hypertrophied cells, where it consists of a network of stout trabeculae united with an inner sheath of primary periosteal bone deposited on the surface of the cartilage, but it thins out and disappears as it approaches the epiphysial cartilage. The nodule is encapsuled by a stout membrane of white fibrous tissue (*f.m.*) which forms the outer boundary of periosteal ossification, and, by moulding the developing periosteal bone, gives a false symmetry to the highly irregular nodule. In the normal embryonic limb endochondral ossification occurs only at the extreme ends of the diaphysis and in the epiphysis. In the ossifying nodule of cartilage at present being described, however, endochondral ossification (*e.b.*) is proceeding throughout the region of hypertrophied cells, the matrix of which is almost entirely calcified; endochondral ossification is most advanced in the regions remote from the areas of epiphysial cartilage.

Several interesting modifications of the usual form of ossification are to be found in certain of the grafts. In graft No. 5 a very large cleft lined by ectodermal epithelium runs right across the growth, and at one point a finger-like

process formed of connective tissue covered by the ectodermal epithelium projects into this cavity for a short distance. This process contains a minute nodule of cartilage on one side of which a thick layer of periosteal bone has been deposited (fig. 10); much of the enclosed cartilage has been excavated and replaced by connective tissue and large multinucleated cells (*g.c.*). The main mass of the periosteal bone is very irregular in shape and is continuous with a complex network of osteogenic fibres (*o.f.*) which extend throughout the connective tissue of the process. A somewhat similar formation of diffuse osteogenic fibres is to be seen in graft 13. Neither of these two nodules of cartilage is enclosed by fibrous membranes such as those described above. Graft 5 shows another interesting abnormality of ossification. A conical ossification centre projecting from a nodule of cartilage is covered in the usual way by a layer of primary periosteal bone. The end of this ossification centre (fig. 11) has completely disintegrated without the agency of inwandering cells. At the tip of the cartilage the matrix forming the capsules has vanished entirely, whilst further back it is found in various stages of dissolution. The degeneration appears to be a process of liquefaction. The normal cartilage matrix in the fowl (Fell, 1925) consists of a dense spongework of delicate fibres and lamellæ which forms stout, well-defined intercellular partitions; the chondroblasts are not in direct contact with these partitions but are connected with them by numerous radiating strands of matrix. In the case at present being described degenerative changes appear first in these radiating strands: the strands fuse together to form strings of liquefying matter which stain deeply with those stains for which cartilage matrix has a special affinity; the strings contract and break their connection either with the cell or with the wall of the capsule, and appear as small horn-like projections on the inside of the capsule or the surface of the cell. They then shrink up into small globules (*gl.*) or round knobs. The wall of the capsule adjacent to the area of liquefaction becomes affected in the same way: the matrix thins out, the surface stains deeply, the partition breaks down and the enclosed cell wanders out. Large portions of the matrix become detached and drift into the cavity where their dissolution continues. Ultimately the matrix vanishes completely, and in the tip of the ossification centre is represented merely by numerous small globules lying amongst the liberated cells. The liberated chondroblasts are of amoeboid shape; many are breaking down, but others, possibly those from the periphery of the cartilage where the cells are usually almost normal in appearance, seem in a healthy state and are occasionally in mitosis (*mi.*). This remarkable form of autolysis also occurs in one of the cartilaginous nodules in graft 13, but in this case is not

associated with ossification. Although only the two cases of autolysis described show this process in its advanced stage, the earliest sign of cartilage disintegration, *i.e.*, the liquefaction of the intracapsular strands to form globules, is to be seen in several nodules in the older grafts.

Large clefts lined by epithelium are present in all the older grafts, and are without exception in a degenerative condition. (Fig. 12.) The superficial cells become keratinized (*k.*), and are cast off into the cavity, whilst large numbers of oxyphil granulocytes migrate between the basal cells and form a thick layer (*g.f.*) between the stratum corneum and the stratum Malpighii; in the older specimens the cavity may be considerably distended by the débris of these elements. The cells of the stratum Malpighii coalesce to form innumerable multinucleated masses of protoplasm (*g.c.*) which present a most striking appearance. As degeneration advances, the cytoplasm of these masses, which usually contains large fat-globules, shrinks greatly and appears crowded with nuclei which are also much shrunken. The nuclei (*n.*) which are usually closely clumped together, gradually become reduced to small, deeply staining bodies, and eventually give rise to a mass of granules lying in the remains of the cytoplasm.

No trace of differentiated muscular tissue was found in the implanted limb-buds. In some of the younger specimens, *i.e.*, grafts of not more than four days' growth, a few large elongated cells, usually unenucleated but sometimes containing several nuclei, are present, either scattered diffusely in the stroma, or, notably in Nos. 2 and 21, arranged in small bundles. The cytoplasm of these elements sometimes presents a fibrillar appearance, and it is possible that they may represent early myoblasts. In the older implants, no sign of muscle at any stage of development could be observed.

The innervation of the grafts seems at best very slight. Comparatively large nerves not uncommonly occur in the substance of the graft, but they appear to have been merely enveloped by the growth of the inoculated tissue and to pass straight through the graft without giving off any branches. Small nerves also accompany many of the small blood vessels passing into the implant, and these are possibly distributed to the tissues of the implant.

Simple reconstructions of eleven of the older grafts were made by means of super-imposed drawings. It was found that the arrangement of the nodules varied greatly in different grafts, and it was impossible to trace any morphological relationship between the nodules and the developing skeleton of the embryonic limb. In Table I are listed the ages and degree of development of the 27 grafts made.

Table I.

No.	Age of Embryo at Time of Amputation of Limb-buds.	Duration of Growth in Chick.	Cartilage.	Bone.	Remarks.
18	81 hours	16 hours	—	—	Consists of undifferentiated mesenchyme.
22	"	"	—	—	" " "
23	"	"	—	—	" " "
14	"	2 days	Present	—	" " "
19	"	"	"	—	
24	"	"	"	—	
26	"	"	"	—	
11	"	3 days	"	—	
16	"	"	"	—	
21	"	"	"	—	
25	"	"	"	—	
2	"	4 days	"	—	
17	"	"	—	—	Graft not established.
20	"	"	Present	—	
27	"	"	"	—	
12	"	5 days	"	—	
7	"	6 days	"	—	
10	"	7 days	"	—	
9	"	8 days	"	—	
6	"	9 days	"	—	
5	"	10 days	"	Present	
15	"	11 days	"	"	
13	"	12 days	"	"	
1	"	15 days	—	—	Graft not established.
3	"	"	?	?	Graft at first grew well, then resorbed.
4	"	"	Present	Present	Largest growth obtained.
8	"	"	"	—	

PART II.—THE DEVELOPMENT OF THE LIMB-BUD WHEN CULTIVATED *in vitro*.1. *Technique*.

(1) *Material*.—The embryos from which the limb-buds were taken were of pure-bred Gold Leghorn (Nos. 1–24) or White Leghorn stock (Nos. 25–44); they had been incubated for 72–80 hours when removed from the egg.

(2) *Method of Cultivation*.—A modification of a tube method which has been used in this laboratory during the past four years was employed.

The medium consisted of a mixture of embryonic extract and plasma diluted, in the earlier experiments, with Compton's and Pannett's isotonic salt solution; the ingredients were mixed in three different proportions approximately as below:

Medium.	Plasma.	Emb. Ex.	Saline.
1	7 drops	2 drops.	50 drops per tube.
2	8 "	8 "	8 "
3	10 "	10 "	0 "

Small centrifuge tubes were employed as culture tubes, and the medium was placed in these and allowed to clot. Medium 1 gave rise to fibrin clots floating in a large quantity of serous fluid; medium 2 formed a soft jelly covered by a layer of supernatant fluid; and medium 3 a tough clot with a mere film of supernatant fluid. An embryo was removed and both the anterior and posterior limb-buds were dissected off from one side of the body—usually the right. The buds on the opposite side were fixed in Allen's solution as controls. The buds to be cultivated were each placed in a separate tube on the surface of the medium and the tube was then tightly corked. In all, 67 cultures were prepared, of which 50 gave satisfactory growths (for analysis of results see Table II). Explants cultivated in medium 1 sometimes adhered to the clot, but more frequently sank to the bottom of the tube, whilst those grown in media 2 and 3 almost always remained on the surface of the fibrin.

The cultures were periodically changed into fresh medium; in the earlier experiments this was done at intervals of two to three days, but in the later half of the work regularly every 48 hours. The explants were removed from the old plasma by means of a wide-mouthed pipette, washed in a drop of saline in a hollow-ground slide, and transferred to one of the new series of culture tubes.

The tube method of tissue culture is very seldom employed. The obvious objection to this mode of cultivation is that changes in the culture cannot be observed in the living condition nearly so well as in tissues growing in a hanging drop. On the other hand, for a histological investigation of organotypic growth *in vitro* it is admirable, since the greater supply of oxygen and food material obtained by this method allows the explant to attain a considerably larger volume without undergoing extensive necrosis. The majority of the cultures grown in media 2 and 3 and changed every 48 hours showed surprisingly little degeneration even in the central regions of the explants and after cultivation for a fortnight or more; in many cases the tissues throughout the growth appeared almost, if not quite, as healthy as in the normal embryonic limb.

(3) *Histological Methods.*—At different periods of growth the explants were removed from the culture tubes with a wide-mouthed pipette and fixed immediately in Allen's modification of Bouin's Fluid warmed to 38° C. Complete serial sections both of the explants and of the control limb-buds were cut, and stained with iron hæmatoxylin, either alone or followed by Van Giesen's stain, hæmatoxylin and eosin, and Mallory's stain.

2. Growth and Differentiation of Explants.

(1) *The Structure of the Limb-bud at the Time of Explantation.*—The size of the limb-buds of embryos of 72–80 hours' incubation is variable and may be greater in an individual of 72 hours than in one of 80 hours. In the following experiments the largest limb-buds employed (from embryos Nos. 7, 9, 22) were of the same size and structure as the majority of the buds used for the grafts, typical examples of which are figured in the text-figure and in fig. 1, whilst the smallest (from embryos Nos. 1, 10, 16, 18, 19, 25, 30, 26) were almost indistinguishable with the naked eye: most of these very minute buds grew and differentiated *in vitro* almost as readily as the larger rudiments. In most cases the limb-buds were intermediate in size between these two extremes. The controls (fig. 13) are found to consist histologically of completely undifferentiated, rather loose mesenchyme, in which are numerous irregular vascular spaces (*b.v.*); even in the largest buds the mesenchyme shows no sign of becoming condensed to form precartilag. The ectoderm is two-layered and along the outer margin is thickened to form a faint ridge (*r*), which is usually less pronounced than in the case of the 82 hours' embryos described in Part I. The anterior bud is very similar in shape and structure to the posterior rudiment, but is slightly smaller.

(2) *Observations on the Living Explants.*—During the first few days of life *in vitro* most of the explants showed "uncontrolled" growth from the cut surface, but later they usually, though by no means invariably, became rounded off and covered by a continuous membrane of connective tissue or epithelium. Liquefaction of the fibrin in the immediate neighbourhood of the explants almost always occurred. In most cases growth proceeded very rapidly until the fifth day, continued rather less actively until the ninth or tenth day, and then diminished until it became inappreciable and in some cases probably stopped altogether. When cultivated in medium 1 the limb-buds, at first solid, after a short time usually became cystic. Sometimes a thin-walled vesicle appeared as a bulge on the surface of the explant, whilst in other cases the entire growth gradually became hollow, giving rise to a uni- or multilocular cyst with delicate translucent walls. This cystic structure usually developed very rapidly, and many of the explants were completely cystic by the third day of growth *in vitro*. Such vesicular cultures sometimes attained a considerable size, and the largest (No. 9 P), which was fixed after 20 days' cultivation *in vitro*, had reached a diameter of about 4 mm. Those buds which remained solid did not enlarge to any great extent. When media 2 and 3 were

employed this cyst formation was not nearly so marked ; only two completely cystic cultures were formed, and the majority of the explants either remained solid or showed slight cyst-formation. Unlike the solid explants grown in a thin medium, those cultivated in the less dilute plasma grew rapidly and sometimes increased to several times their original size. As differentiation proceeded cartilage began to form, and the cartilaginous nodules could usually be faintly distinguished.

(3) *The Histological Development of the Explants.*

(a) *Histology of cultures grown in a thick medium.*—As stated in the description of technique, two types of dense medium were employed, one more solid than the other, but as both gave essentially the same results the growths will be described in a single series.

In the younger explants, *i.e.* in those which have been cultivated for not more than four days *in vitro*, it is usually possible to distinguish roughly the original contour of the limb-rudiment, and to determine which was the proximal and which the distal region of the bud owing, in the first place, to the persistence for the first few days of cultivation *in vitro* of that ridge of thickened ectoderm which runs along the outer margin of the bud, and, in the second place, to the fact that the cut surface of the bud usually becomes attached to the clot of fibrin by the outwandering of the mesenchyme cells, and is, therefore, not covered with ectoderm. The first signs of tissue differentiation appear after two days' growth *in vitro*. In two of the three specimens fixed at this age the mesoderm composing the proximal* part of the limb-bud has condensed into an oblong mass of cells representing precartilage (fig. 14). This elongated mass is slightly broader at one end than at the other, the larger end lying near the cut surface of the limb-bud. It is interesting to note that as regards both shape and position in the limb-bud this block of precartilage resembles the earliest rudiment of the posterior appendicular skeleton in the normal embryo. In one specimen another small, more or less spherical nodule is situated near the larger end of the main piece of precartilage. The general stroma of the explants is composed of a network of stellate cells, as in the normal early limb. There is comparatively little cell degeneration, and mitotic figures are very numerous in both the mesoderm and ectoderm. The vascular sinuses are seen as irregular branching cavities and are considerably distended.

After three days' cultivation *in vitro* the cells occupying the central region

* The words "proximal" and "distal" are used with reference to the original relationship of the limb-bud to the embryo.

of the precartilaginous mass become less densely packed together, enlarge somewhat and at the same time become slightly flattened in a direction at right angles to the long axis of the mass; at the periphery the precartilage fades into undifferentiated mesenchyme. Between the stellate cells of the loose connective tissue composing the rest of the explant a delicate intercellular network of amorphous material now appears, which has the staining reactions of white fibrous tissue.

By the fourth day of cultivation the greater part of the precartilage has assumed the character of early cartilage. In the single specimen (fig. 15) fixed at this stage of development the skeletal mass is broken up into a series of oblong segments (c. 1., c. 2, and p.c.). One of the nodules (c. 1) is situated at the extreme end of the explant, in what appears to have been originally the proximal portion of the limb-bud, and consists of fairly well developed cartilage. A broad middle region, occupying about half the total length of the block is composed of cells, slightly flattened in a direction parallel with the long axis of the cartilage, whilst the terminal regions contain rounded or polyhedral chondroblasts. The cartilage fades imperceptibly into mesenchyme and the matrix consists only of a few fine strands and lamellæ running between the cells. The adjacent cartilaginous segment (c. 2) is also comparatively well differentiated towards the proximal end, but about half-way along its length it is still at the precartilaginous stage. Two other small skeletal masses are present in this culture which are not shown in the figure. The histological structure of the larger cartilaginous nodules in this specimen is almost identical with that of the posterior appendicular skeleton in a normal embryo after four days' incubation. The principal difference lies in the fact that in the normal embryo the rudiments of a perichondrium are seen, whilst in the growth there is no sign of such a structure. Chondrogenesis in the explant also resembles the process in the normal embryonic limb in that chondrification is most advanced in what was originally the basal region of the limb-bud.

In cultures which have been growing *in vitro* for five or six days the cartilage is found to have reached the "epithelioid" stage of development described on p. 344. The ectoderm has also undergone a certain amount of differentiation, and the superficial cells show commencing keratinisation. At this stage, also, various areas of the ectoderm begin to show a peculiar structure, which occurs to some extent in almost all the limb-bud cultures, sometimes (fig. 17) on a very extensive scale, and is also figured by Maximow (1925). In the regions to which we refer the basal columnar cells consist of a single stratum, but several layers of superficial flattened cells have been

formed, and give rise to a complex system of small cysts varying greatly in size, the walls of which consist of either one or two layers of flattened cells. The way in which these cysts arise appears to be as follows: numerous small clefts containing fluid are formed between the superficial strata; the clefts gradually become distended into small round cavities, and finally a foam-like mass of thin-walled vesicles is formed.

By the seventh day after explantation the histological structure of the cartilage shows a comparatively advanced stage of differentiation. Unlike the epithelioid cartilage of the previous stage, the cells are separated by broad partitions of matrix; the chondroblasts themselves, however, show little structural change; many of the cells are in mitosis. The cartilage near the periphery of the nodules is of the epithelioid type but has a fairly distinct outline, and does not merge into a mass of undifferentiated mesenchyme as in the younger cultures.

After the seventh day of growth *in vitro*, the limb-buds undergo little further development, although they may continue to exist in a comparatively healthy condition for another fourteen days. Mitosis usually occurs to some extent, even in the oldest cultures (fig. 18), but as a rule diminishes in amount after the tenth or eleventh day of growth. In the oldest cultures there is usually a greater quantity of matrix present, but otherwise the histological structure of the cartilage shows little change. In the older specimens many of the nodules are partially or completely covered by a perichondrium (fig. 21, *pch.*) composed entirely of white fibrous tissue, which on the inside fades into the cartilage. As the period of cultivation lengthens the amount of white fibrous tissue in the stroma increases, the delicate intercellular web of amorphous material giving rise to a close network of white fibres (fig. 24). In some of the growths one or more small very dense masses of fibrous tissues are present (fig. 19, *f.t.*). The keratinisation of the epidermis continues, and the explants become invested by a stratified coat of horny material which becomes progressively thicker as the age of the culture advances (fig. 23, *k.*).

The shape and arrangement of the precartilaginous and cartilaginous nodules in different limb-bud cultures is by no means constant. In most cases, however, it is found to conform to one of three general types. The first and most common of these, which might be termed the "axial" type, is very well illustrated by the four days' old culture (fig. 15) described above. In such cases the cartilage consists of (1) a single rod, or (2) of two or three elongated cartilages set end to end, or (3) of an elongated cartilage at one end of which lie one or more irregular nodules (figs. 19 and 21). In the second type of arrangement two

rounded nodules of equal or unequal size lie side by side, and in the third type a single large nodule is present (fig. 20). In a few of the explants the distribution of the nodules is quite irregular. In some of those specimens in which the axial configuration of the nodules is most marked it is found that the degree of differentiation of the cartilage is considerably more advanced at one end of the explant than at the other. Fig. 21 represents a section of such a specimen.

The ectoderm in many of the cultures gives rise to cords and solid tubules (fig. 21, *ep.t.*) running into the substance of the growth. Some of the cords become transformed into epithelial cell nests (fig. 22). These bodies are solid spheres of tissue composed of concentric layers of flattened epithelial cells. The epithelial cords are at first slender structures continuous at one end with the superficial ectoderm. A cord which is destined to give rise to cell nests becomes thickened at one or more points, the cells in these places assuming a concentric arrangement; this results in a contraction in the length of the cord, and the connection with the epidermis becomes more and more slender and finally breaks; an increasing number of cells become concentrated about the node or nodes until at length the whole cord is condensed into one or more independent cell nests. In one case three such nests are in course of formation from a single rather stout cord. In the oldest cultures the epithelial cords and cell nests usually become wholly transformed into keratin, the process beginning in the interior and spreading outwards towards the periphery. In one specimen (fig. 16) part of the ectoderm shows a rather remarkable structure: a single superficial layer of flattened cells is present (*s.s.*) but the basal stratum (*b.s.*) instead of forming a continuous sheet of tissue one cell deep, has given rise to a mass of solid tubules or trabeculae forming a thick layer beneath the superficial flattened cells. The cords for the most part run in a direction parallel with the surface of the explant, but a few penetrate far into the interior; they have no basement membrane, are set very closely together and repeatedly anastomose. Several of the cultures are completely or partially devoid of an epidermal covering and contain instead a large ectodermal sac entirely enclosed by the connective tissue of the explant (fig. 15). The epithelium undergoes keratinisation in the usual way, and in the oldest growths in which such sacs occur the cavities are filled with large quantities of keratin, whilst in places the entire epithelial wall is composed of flattened cells in all stages of horny infiltration. These sacs appear to be formed as follows. Instead of growing over the cut surface, part of the ectoderm accompanied by a layer of the subjacent mesenchyme bends backwards and grows over the surface of the

explant, eventually fusing at the edges with the underlying epidermis, thus forming a closed cavity. In some cases the entire ectoderm, together with a layer of mesenchyme, becomes reflected upon itself in this manner; the edges of the overgrowing sheet ultimately meet and fuse and a single large sac is formed, the epithelial lining of which represents the entire ectoderm of the limb-bud, which is thus left totally devoid of an epidermal covering.

Necrotic areas may occur even in very young cultures, but in the majority of explants this degeneration is remarkably slight and in many is almost absent (in this connection it may be remarked that small degenerative regions also occur in the normal limb during early development (Fell, 1925, p. 429)). Necrosis of cartilage somewhat resembles the autolysis described in certain of the grafts (p. 348); the surrounding stromal cells take no part in the destruction of the cartilage, and even in those regions where degeneration is most complete the original contour of the nodule is preserved. In the case of the explants, however, the liberated chondroblasts are completely degenerate. The stroma may also show degenerative areas.

Many of the explants are partially and two completely cystic. These cysts are formed in three ways: (a) by the accumulation of fluid between the ectoderm and mesoderm, so that the ectoderm becomes distended to form a large, blister-like vesicle on the surface of the culture; (b) by the distension of the embryonic blood vessels, which thus give rise to a number of large rounded cavities communicating with one another and lined by endothelium; (c) by a local necrosis in the mesoderm, the cavity thus formed becoming greatly swollen by the accumulation of the fluid. In some specimens all three types of cyst-formation are to be seen in the same explant.

(b) *The histology of cultures grown in a thin medium.*—The cultures grown in a thin medium differ from those described in the preceding section (1), in showing a greater tendency to cyst-formation; (2) in the comparatively poor development of cartilage; (3) in the relatively slight keratinisation of the epithelium investing the older cultures, and (4) in that all the tissues are more liable to degeneration.

Of the sixteen successful cultures, three only are solid, four are partially cystic and nine completely cystic. The three modes of cyst-formation described in the preceding section all occur in these specimens. A study of the younger cultures indicates that a completely cystic condition of the explant originates at an early period of development *in vitro* by one or more of the above processes. Fig. 25 shows a completely cystic growth which had been cultivated for three days. Slightly older cystic cultures of ages ranging from

Table II.

	No. of Culture.	Duration of Growth.	No. of times changed into fresh medium.	Cartilage.	Remarks.
		Days.			
Medium III.	27 A	1	0	absent	
	27 P	2	0	precartilage	
	37 A	2	0	"	
	34 A	3	1	present	
	37 P	4	1	"	
	35 A	5	2	"	
	36 A	6	2	"	
	36 P	7	3	"	
	35 P	8	3	"	
	31 P	8	3	—	rejected.
	29 A	9	4	present	
	26 P	10	4	"	
	32 A	11	5	"	
	31 A	12	5	"	degenerated.
	25 A	13	6	"	
	29 P	14	6	"	
	28 A	15	7	"	
	33 A	16	7	"	
	26 A	17	8	"	
	34 P	18	8	"	
	28 P	19	9	"	
	30 A	20	9	"	
	32 P	21	10	"	
	33 P	22	10	"	
Medium II.	22 P	2	0	absent	
	24 A	2	0	—	rejected.
	18 A	10	4	present	
	19 A	10	4	"	
	20 P	10	4	"	
	23 P	10	4	"	completely cystic.
	18 P	11	4	"	
	19 P	11	4	"	
	20 A	11	4	absent	very poor growth.
	21 A	11	4	present	
	21 P	11	4	"	
	22 A	11	4	"	completely cystic.
	23 A	11	4	"	
	24 P	11	4	"	
Medium I.	9 A	2	0	absent	
	2 A	3	0	"	completely cystic.
	7 P	3	0	"	
	2 P	4	1	"	completely cystic.
	15 P	4	0	"	
	7 A	5	1	"	" "
	14 P	5	1	"	" "
	1 P	6	1	"	
	5 A	6	1	present	
	5 P	6	1	—	rejected.
	12 P	6	1	—	"
	13 P	6	1	—	lost through accident.
	8 A	8	2	absent	" " "
	3 A	10	3	present	
	12 A	10	2	absent	
	13 A	10	2	—	rejected.

Table II (continued).

	No. of culture.	Duration of Growth.	No. of times changed into fresh medium.	Cartilage.	Remarks.
		Days.			
Medium I.	15 A	10	2	present	completely cystic.
	16 A	10	2	"	
	16 P	10	2	absent	
	3 P	11	3	present	completely cystic.
	10 P	12	3	absent	
	11 P	12	3	present	
	6 P	13	3	"	lost through accident.
	4 A1	13	3	"	
	6 A	19	4	—	
	4 P	20	4	absent	completely cystic. completely cystic.
	4 A2	20	4	present	
	8 P	20	4	absent	
	9 P	20	4	present	completely cystic. completely cystic.
	10 A	20	4	"	

three to six days may be simple or multiple cysts; the cyst wall is for the most part composed of greatly attenuated epithelium and a few thin layers of connective tissue. After the ninth or tenth day of cultivation, the cyst wall is found to be stouter and of tougher texture, owing to an increased development of white fibrous tissue, and one or more nodules of early cartilage are sometimes present embedded in and continuous with the white fibrous tissue. The largest specimen obtained (fig. 26) which was fixed after 20 days' growth, attained a diameter of about 4 mm.; it consists of a single chamber and three nodules of cartilage are present in the tough fibrous wall.

Of the solid and partially cystic explants only two contain healthy cartilage, although several show small necrotic nodules.

The ectoderm of the limb-buds cultivated in a thin medium in every case completely envelops the growth. Keratinisation, though never very striking, occurs to some extent in all the older explants, but even in cultures of twenty days' growth, the horny infiltration is seldom so great as completely to obliterate the internal structure of the cells. With one notable exception the superficial cysts formed from the outer layers of flattened cells, although usually present, are much less conspicuous than in the explants grown in a thick medium.

Discussion.

There are two principal differences between the histological composition of the growths and that of the normal limb, viz., the absence of differentiated muscle in both grafts and cultures and the absence of bone in the cultures.

The writers are at present not in a position to discuss the possible causes of the non-development of muscle and bone under these experimental conditions.

Among the minor histological abnormalities found in the grafts and cultures may be mentioned the curious autolysis of cartilage described on pp. 348-349, in which the matrix is liquefied apparently by the chondroblasts themselves. In the case of the explants the process is evidently of a necrotic nature, as shown by the degenerative character of the cells. In the grafts, however, the cartilage cells in the unaffected portions of the nodule, though hypertrophied and showing no mitosis, nevertheless appear in a fairly healthy condition, whilst many of those lying in the liquefied matrix are perfectly normal in structure and may even exhibit phases of mitotic division. It is interesting to note that we have here a phenomenon almost exactly corresponding to the behaviour of differentiated cartilage when cultivated *in vitro*, as described by Fischer (1922).

It remains to discuss the important question: Does the gross anatomical structure of the skeletal tissue in the grafts and explants correspond with that of the normal limb-skeleton? In the case of the grafts there appears to be no resemblance either in shape or mutual arrangement between the cartilage nodules of the implants and the developing cartilage-bone of the embryonic limb. This is also true of the cystic explants cultivated in medium 1 and of certain of the cultures grown in media 2 and 3. In 6 of the younger and 9 of the older explants cultivated in media 2 and 3, however, the precartilage or cartilage—whichever happens to be present—shows what we have termed an axial arrangement; in such cases it would seem that a morphological relationship does undoubtedly exist between the skeletal tissue of the explants and of the normal limb. As in the normal limb-rudiment (Johnson, 1883) an elongated condensation of mesenchyme appears in what was originally the proximal region of the explanted bud, and gradually extends towards the distal part; this mass usually becomes subdivided into two and sometimes three segments, segmentation beginning in the proximal part of the bud, as in the embryo. In the explants, however, the gross morphological development proceeds no further although the cartilage continues to differentiate histologically for some time longer. This arrest of the anatomical development of the skeleton in the explanted limb-bud may be explained as follows. In the cultures growth and differentiation both proceed at considerably less than the normal rate. During the first three days of life *in vitro*, growth and differentiation lag behind the normal to about the same extent. Soon, however, it is found that, as compared with normal development, the

process of tissue differentiation rapidly outstrips that of growth ; for instance, the degree of histological differentiation of the cartilage in an explant of eleven days' growth may approximately correspond with that of the cartilage in the limb of an embryo of five days' incubation, although the explant itself is only one-third the size of the five days' old limb. In the culture, therefore, the precartilaginous rudiment of the limb-skeleton, owing to its extremely slow growth, becomes completely chondrified long before it has had time to elongate and complete its segmentation in the normal way.

This disparity between the rate of growth and the rate of differentiation probably accounts for the fact that the three cellular zones, seen in the normal limb-cartilage and in many of the nodules in the grafts, are not found in a single nodule in the explants. In the normal cartilage chondroblastic hypertrophy first appears in the middle of the shaft (the chondrification centre) and spreads outwards ; whilst the most actively proliferating and least highly differentiated regions are the distal ends. The intermediate elements are thus subjected to pressure by the enlargement of these two regions, and become flattened. In the slowly differentiating and still more slowly growing explants, however, pressure due to rapid regional growth must be absent or inappreciable. When inoculated into the subcutaneous tissue of the chick, on the other hand, the limb-buds do not lag behind the normal either in rate of growth or in rate of tissue differentiation to nearly the same extent as when cultivated *in vitro*, and it is probably because of this that the three cellular zones seen in the normal limb are frequently found in the cartilage nodules of the grafts.

The fact that in no single instance does the skeletal tissue of the grafts correspond in gross anatomy with the normal limb-skeleton is probably due to the inability of the implant to grow freely when embedded in the tissues of another animal. The developing limb-bud would encounter on all sides abnormal pressures and resistances which must inevitably distort the growing cartilage out of all semblance to its normal morphological structure. The limb-buds growing *in vitro*, on the contrary, in favourable experiments do not suffer from this disadvantage, but either lie loosely in a small cavity of liquid medium or, in the earlier stages, are attached to the fibrin clot by the cut surface only, *i.e.*, by the end normally continuous with the body-wall of the embryo. The cases in which the skeletogenous tissue of the explanted limb-buds fails to develop along normal lines may probably be attributed to one or more of the following causes : (1) to the distortion of the original arrangement of the mesenchyme by internal cyst formation or by ingrowth of an epithelial sac ; (2) to the explantation of an incomplete or damaged bud ; (3) to placing

the bud upside down on the surface of the clot; (4) to the bud being closely embedded in fibrin during some stage of development.

The researches of Braus, Harrison (1907, 1918, 1921) and others have shown conclusively that in the Amphibia the undifferentiated limb-bud constitutes a self-differentiating equipotential system. In the case of the fowl the results of the present investigation indicate that the undifferentiated limb-rudiment possesses a considerable capacity for self-differentiation. With the exception of muscle and bone, the tissues normally present in the embryonic limb also appear in the cultures, undergoing a remarkable degree of histological development in the complete absence of a blood and nerve supply, whilst in favourable cases the skeletogenous tissue even shows the earlier stages in the formation of a normal limb-skeleton. The writers' experiments, however, shed no further light on the question as to whether the limb-bud of the fowl is also an equipotential system, and a discussion of this point is therefore beyond the scope of the present communication.

Summary.

1. The undifferentiated limb-buds of fowl embryos of 82 hours' incubation were inoculated subcutaneously into the under-surface of the wings of post-embryonic chicks.

2. The grafts grew well and differentiated into cartilage, bone, fibrous tissue and typical epidermis: differentiated muscle was absent.

3. The general morphology of the skeletal tissue in the grafts bore apparently no relation to that of the normal limb-skeleton.

4. The undifferentiated limb-buds from fowl embryos of 72-80 hours' incubation were cultivated *in vitro* by means of an improved tube technique.

5. The explants showed active cell-division and cartilage, white fibrous tissue and typical epidermis differentiated during the period of cultivation.

6. When grown in a relatively fluid medium many of the cultures developed into large balloon-like cysts; in such cases the shape and arrangement of the cartilaginous nodules showed no correspondence with the structure of the developing cartilage-bones of the normal limb.

7. When a more solid culture medium was employed cyst-formation was far less pronounced, and in explants cultivated under such conditions the skeletogenous tissue frequently showed the early stages in the development of a normal limb-skeleton.

REFERENCES TO LITERATURE.

- Chlopin, N., "Ueber '*in vitro*' Kulturen der embryonalen Gewebe der Säugetiere," 'Arch. f. Mik. Anat.,' vol. 96, p. 435 (1922).
- Detwiler, S. R., "Experiments on the Development of the Shoulder Girdle and the Anterior Limb of *Amblystoma punctatum*," 'Jl. Exp. Zool.,' vol. 25, No. 2, p. 499 (1918).
- Idem.*—"Co-ordinated Movements in Supernumerary Transplanted Limbs," J. Comp. Neur., vol. 38, No. 4, p. 461 (1925).
- Fell, H. B., "The Histogenesis of Cartilage and Bone in the Longbones of the Embryonic Fowl," 'J. Morph.,' vol. 40, No. 3 (1925).
- Fichera, G., "Développement des greffes embryonnaires et fœtales," 'Arch. de Méd. Exp.,' vol. 21, p. 615 (1909).
- Fischer, A., "A Pure Strain of Cartilage Cells *in vitro*," 'J. Exp. Med.,' vol. 36, No. 4, p. 379 (1922).
- Idem.*—"Cultures of Organised Tissues," 'J. Exp. Med.,' vol. 36, No. 4, p. 393 (1922).
- Harrison, R. G., "Experiments in Transplanting Limbs and their Bearing upon the Problems of the Development of Nerves," 'Jl. Exp. Zool.,' vol. 4, No. 2, p. 239 (1907).
- Idem.*—"Experiments on the Development of the Fore-limb of *Amblystoma*, a Self-Differentiating Equipotential System," 'Jl. Exp. Zool.,' vol. 25, No. 2, p. 413 (1918).
- Idem.*—"On Relations of Symmetry in Transplanted Limbs," 'Jl. Exp. Zool.,' vol. 32, No. 1, p. 1 (1921).
- Johnson, A., "On the Development of the Pelvic Girdle and Skeleton of the Hind Limb in the Chick," 'Q. Jl. Micr. Soc.,' vol. 23, p. 399 (1883).
- Maximow, A., "Tissue Cultures of Young Mammalian Embryos," 'Contribs. Embryol.,' vol. 16, No. 80, p. 49 (1925) (Carneg. Inst., Wash.).
- Murray, P. D. F., and Huxley, J. S., "Self-Differentiation in the Grafted Limb-bud of the Chick," 'Jl. Anat.,' vol. 59, No. 4, p. 379 (1925).
- Nicholas, J. S., "Ventral and Dorsal Implantations of the Limb-bud in *Amblystoma punctatum*," 'Jl. Exp. Zool.,' vol. 39, No. 1 (1924).
- Idem.*—"Regulation of Posture in the Fore Limb of *Amblystoma punctatum*," 'Jl. Exp. Zool.,' vol. 40, No. 1, p. 113 (1924).
- Rous, P., "An Experimental Comparison of Transplanted Tumour and a Transplanted Normal Tissue capable of Growth," 'Jl. Exp. Med.,' vol. 12, No. 3, p. 344 (1910).
- Idem.*—"The Effect of Pregnancy on Implanted Embryonic Tissues," 'J. Exp. Med.,' vol. 13, No. 2, p. 248 (1911).
- Shattock, S. G., Seligman, C. G., and Dudgeon, L. S., "An Attempt to produce Chondromatous or Osteomatous Growth by the Grafting of Fœtal Bones," 'Proc. Roy. Soc. Med. Lond.,' vol. 3, Path. Sec., p. 127 (1909).
- Spurling, R. G., "The Effect of Extirpation of the Posterior Limb-bud on the Development of the Limb and Pelvic Girdle in Chick Embryos," 'Anat. Rec.,' vol. 26, No. 1, p. 41 (1923).
- Thomson, D., "Some Further Researches on the Cultivation of Tissues *in vitro*," 'Proc. Roy. Soc. Med. Lond.,' vol. 7, Pt. I, 'Marc. Beck. Lab. Reps.,' p. 21 (1914).
- Idem.*—"Controlled Growth *en masse* (Somatic Growth) of Embryonic Chick Tissue *in vitro*," 'Proc. Roy. Soc. Med. Lond.,' vol. 7, Pt. I; 'Marc. Beck. Lab. Reps.,' p. 71 (1914).

DESCRIPTION OF PLATES.

Abbreviations.

<i>am.</i> = amnion.	<i>gl.</i> = globule of matrix.
<i>ba.</i> = base of limb-bud.	<i>gr.</i> = granulocyte.
<i>b.cl.</i> = blood clot.	<i>g.t.</i> = graft tissue.
<i>b.s.</i> = basal stratum.	<i>h.t.</i> = host tissue.
<i>b.v.</i> = blood vessel.	<i>k.</i> = keratin.
<i>c.</i> = cartilage.	<i>l.t.</i> = lymphoid tissue.
<i>ck.</i> = cork.	<i>m.</i> = muscle.
<i>cy.</i> = cyst.	<i>mi.</i> = mitosis.
<i>e.b.</i> = endochondral bone.	<i>m.s.</i> = marrow space.
<i>e.c.</i> = ectodermal cord.	<i>n.</i> = nucleus.
<i>ect.</i> = ectoderm.	<i>o.c.t.</i> = outwandering connective tissue cells.
<i>ep.</i> = epidermis.	<i>o.f.</i> = osseous fibre.
<i>ep.r.</i> = epiphysial region.	<i>p.b.</i> = periosteal bone.
<i>ep.t.</i> = epithelial tube.	<i>pch.</i> = perichondrium.
<i>f.m.</i> = fibrous membrane.	<i>r.</i> = terminal ridge of ectoderm.
<i>f.t.</i> = fibrous tissue.	<i>s.s.</i> = superficial stratum.
<i>g.</i> = graft.	<i>z.f.c.</i> = zone of flattened cells.
<i>g.c.</i> = giant cell.	<i>z.h.c.</i> = zone of hypertrophied cells.
<i>g.f.</i> = granulocytic débris.	

PLATE 5.

FIG. 1.—Section of left posterior limb-bud of an 82 hours' embryo (No. 7). Note the completely undifferentiated character of the mesenchyme and the thickened terminal ridge (*r.*) of ectoderm. (*N.B.*—The ectoderm has become slightly detached from the mesenchyme during dissection.) (Iron Hæm.)

FIG. 2.—Limb-bud (No. 18) (*g.*) 16 hours after inoculations into the subcutaneous tissue of a post-embryonic chick. The host tissue is somewhat inflamed and the scar (*b.cl.*) where the implant was inserted is visible. (*N.B.*—Figs. 2, 7 and 9 are drawn to the same scale.) (Hæm. Eos.)

FIG. 3.—Mesenchyme of graft No. 18, as seen under high power. Note mitosis (anaphase and prophase) and infiltration of granular cells (*gr.*). (Hæm. Eos.)

FIG. 4.—Developing ectodermal cord in graft No. 18. (Hæm. Eos.)

PLATE 6.

FIG. 5.—Epithelioid cartilage from graft (No. 26) of two days' growth. Note the mitotic figures (metaphase and prophase) and the narrow intercellular partitions of matrix (Mallory's stain.)

FIG. 6.—Epithelial islet from graft (No. 7) of 6 days' growth, showing formation of cavity and active cell division. (Iron Hæm. Van Gies.)

FIG. 7.—Graft (No. 7) of 6 days' growth. Nodules of cartilage and epithelial tubules (*ep.t.*) are present. Cf. fig. 1, which represents the corresponding bud from the opposite side of the same embryo. (Mallory's stain.)

FIG. 8.—Nodule of cartilage from 10 days' old graft (No. 5) from the same embryo, showing the three cellular zones: zone of hypertrophied cells (*z.h.c.*), zone of flattened cells (*z.f.c.*) and epiphysial region (*ep.r.*). (Mallory's Stain.)

PLATE 7.

- FIG. 9.—Portion of graft No. 4 of 15 days' growth. The cartilage shows periosteal (*p.b.*) and endochondral ossification (*e.b.*) and contains irregular marrow spaces (*m.s.*). The large nodule is encapsuled by a fibrous membrane (*f.m.*). (Mallory's stain.)
- FIG. 10.—Diffuse ossification seen in graft No. 5. Note the osseous fibres (*o.f.*) running out from the main mass of bone into the surrounding stroma; several large giant cells (*g.c.*) are present. (Mallory's stain.)

PLATE 8.

- FIG. 11.—Autolysis of cartilage in graft No. 5. The matrix is breaking down and in places is reduced to globules (*gl.*). Note the amœboid form of the liberated chondroblasts, one of which has rounded off and is undergoing mitosis (*mi.*). (Iron Hæm. Van Gies.)
- FIG. 12.—Portion of epithelial tubule from graft No. 8 of 15 days' growth, showing advanced stage of degeneration. The cells of the basal layer have fused into large multinucleated masses of protoplasm (*g.c.*), whilst the superficial cells are completely keratinised (*k.*). Between the two layers is a mass of granulocytic debris (*g.f.*). (Iron Hæm.)

PLATE 9.

- FIG. 13.—Transverse section through posterior trunk region of embryo of 72 hours (No. 29). On the right-hand side of the figure is seen a section of the left posterior limb-bud; the right posterior limb-bud has been amputated for explantation *in vitro*, and is shown in fig. 19 after 14 days' cultivation. (*N.B.*—Figs. 13, 14, 15, 19, 25 and 26 are drawn to the same scale.) (Hæm. Eosin.)
- FIG. 14.—Anterior limb-bud (No. 37A) after two days' growth *in vitro*. Shows elongated mass of precartilage (*p.c.*) dilated blood vessels (*b.v.*) and terminal ridge of ectoderm (*r.*). (Hæm. Eos.)
- FIG. 15.—Posterior limb-bud (No. 37P) after four days' cultivation *in vitro*. The specimen here figured is from the same embryo as the explant (No. 37A) represented in fig. 14. Note the two nodules of early cartilage (*c. 1* and *c. 2*), showing the "axial" type of arrangement, the ectodermal sac (*ect.*), and mass of fibrous tissue (*f.t.*). (Mallory's Stain.)
- FIG. 16.—Ectoderm from explant No. 35A, showing the formation of loose cords by the basal layer (*b.s.*); the superficial stratum (*s.s.*) is normal. (Hæm. Eos.)

PLATE 10.

- FIG. 17.—Ectoderm from limb-bud (No. 20P) of 10 days' cultivation showing the complex froth-like system of cysts formed by the flattened cells of the superficial layer. (Iron Hæm. Van Gies.)
- FIG. 18.—Cartilage cells from explant of 21 days' growth. The nucleus of one of the cells is in anaphase. (Iron Hæm.)

PLATE 11.

- FIG. 19.—Right posterior limb-bud (No. 29P) after 14 days' cultivation. *Cf.* fig. 13, which shows the left posterior (control) limb-bud from the same embryo, and note the extensive differentiation which has taken place *in vitro*. The cartilage nodules in 29P show the "axial" form of arrangement. Note the cystic cavity (*cy.*) dense mass of fibrous tissue (*f.t.*) and keratin (*k.*). (Iron Hæm.)
- FIG. 20.—Explant (No. 26P) after 10 days' cultivation. This culture is firmly embedded in the plasma owing to the outwandering (uncontrolled growth) of the connective tissue (*o.c.t.*); a single nodule of cartilage is present; there is no epithelial covering. (Iron Hæm. Van Gies.)

PLATE 12.

- FIG. 21.—Portion of 11 days' culture (No. 20A). The nodules of cartilage show the "axial" arrangement; the chondroblasts of nodule *c. 2* are hypertrophied. Note the thick, fibrous perichondrium (*pch.*) and epithelial tubules (*ep.t.*). (Mallory's stain.)
- FIG. 22.—An epithelial cell-nest from explant No. 23A. (Iron Hæm. Van Gies.)
- FIG. 23.—Ectodermal epithelium from culture No. 25A of 13 days' growth showing thick layer of keratin (*k.*). (Mallory's stain.)
- FIG. 24.—Stroma from explant (No. 33P) of 22 days' cultivation showing dense network of fine white fibres. (Mallory's stain.)

PLATE 13.

- FIG. 25.—Early cyst-formation owing (probably) to extreme dilation of blood vessels in a culture (No. 2A) of 3 days' growth in medium 1. Note the terminal ridge of the thickened ectoderm (*r.*). (Iron Hæm.)
- FIG. 26.—Portion of largest cyst obtained (No. 9P); about half the section is shown in this figure. (*Cf.* fig. 13, drawn to the same scale.) Two nodules of cartilage (*c.*) are present in one of which is embedded a small piece of cork (*ck.*). This explant was cultivated for 20 days in medium 1. (Mallory's stain.)

The Meiotic Phase in Certain Mammals.

By C. E. WALKER, D.Sc., Department of Cytology, University of Liverpool.

(Communicated by J. B. Farmer, F.R.S.—Received December 23, 1925.)

(PLATES 14–16.)

Cytological literature is very voluminous and is scattered in many journals of varying importance published in different languages. It may be, therefore, that the resemblance between certain stages of the early telophase of cell division in some cells and the stages in the 1st Meiotic Division, very commonly known as "synaptic," has been recorded on several occasions. I have, however, only found one definite reference to this similarity (Blackman, 1903).

If the observations recorded here and my interpretation of them are correct, this similarity should be of usual occurrence. That the daughter chromosomes of a somatic division frequently show longitudinal fission has, as I have pointed out on a previous occasion, been observed frequently. (Walker, 1925.) Flemming called it "precocious longitudinal division" as long ago as 1891.*

According to the observations here recorded, the chromosomes massed

* For further references see Walker, C. E., 'Roy. Soc. Proc.,' B, vol. 98 (1925).



Fig. 1

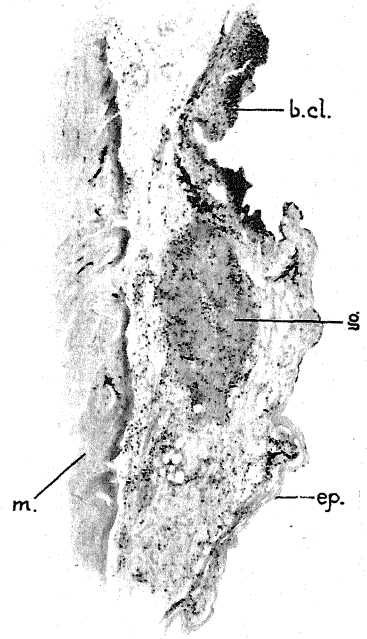


Fig. 2

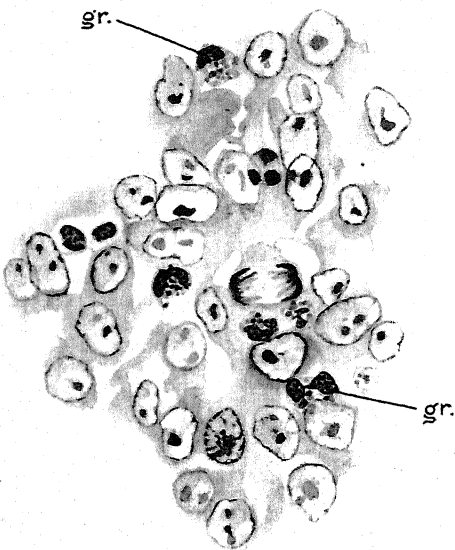


Fig. 3

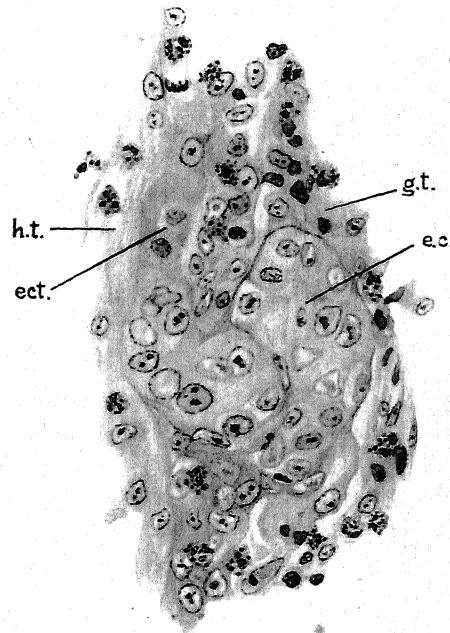


Fig. 4

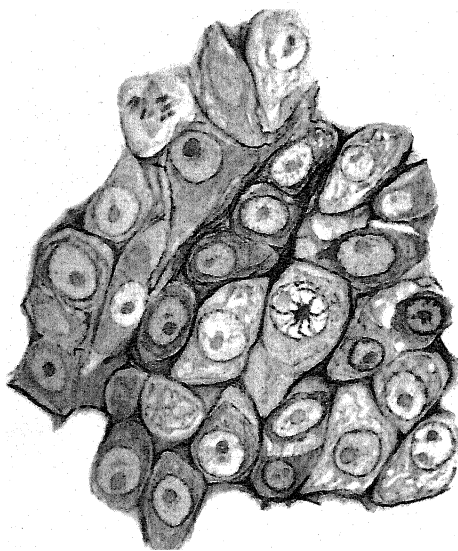


Fig. 5

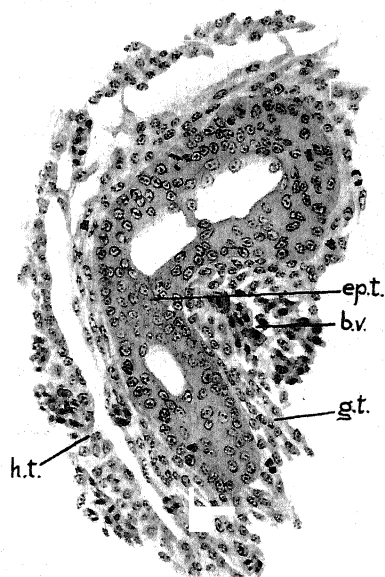


Fig. 6

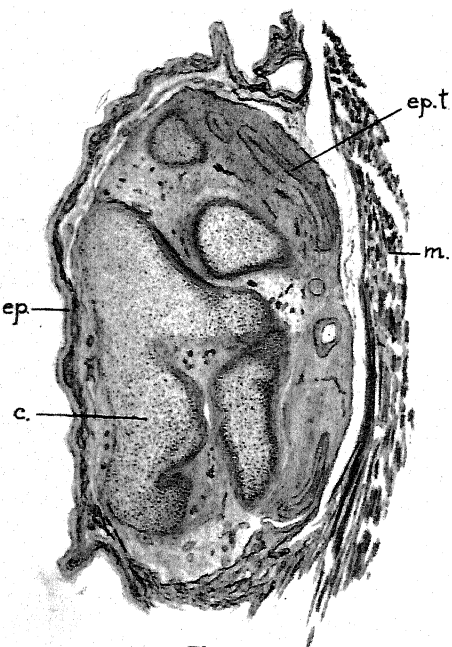


Fig. 7

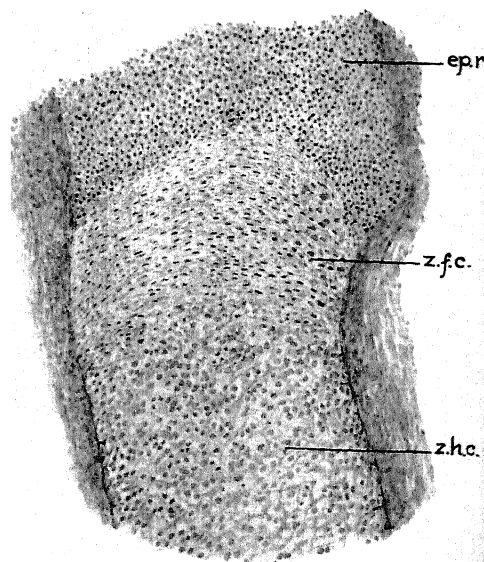


Fig. 8

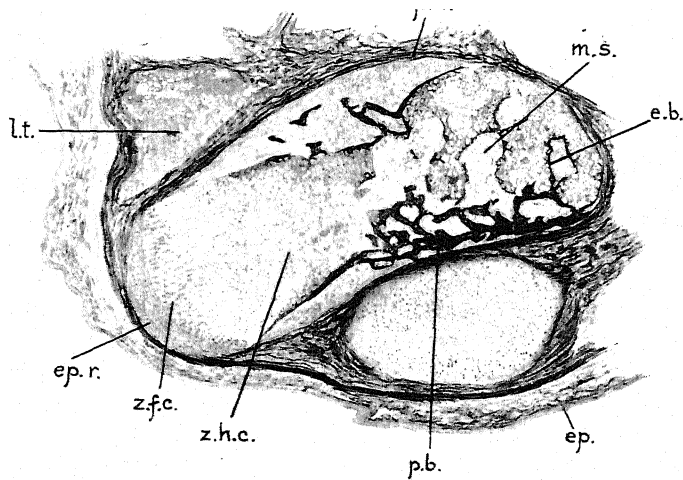


Fig. 9

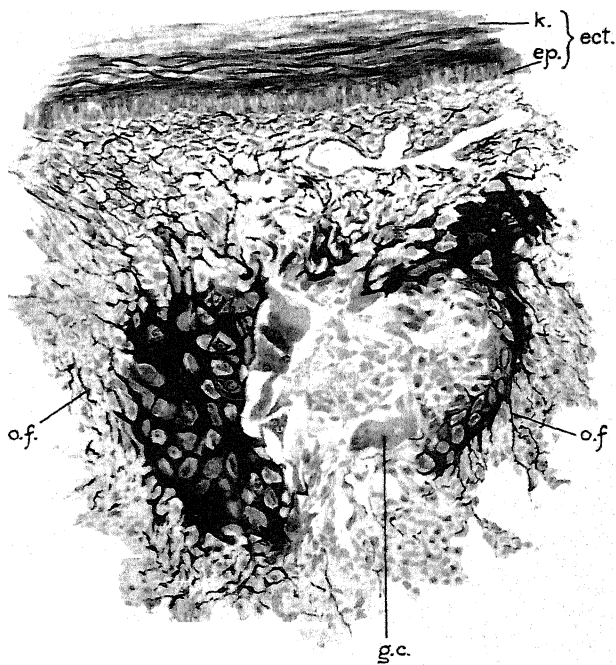


Fig. 10

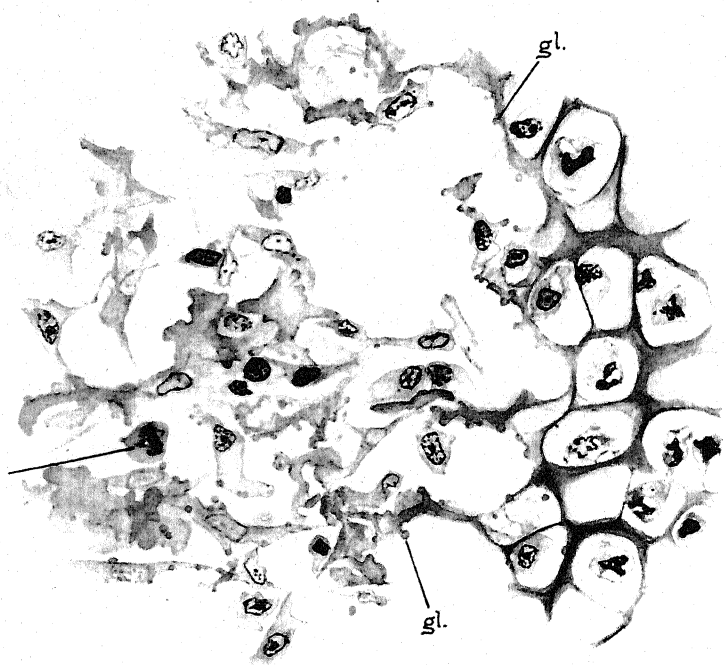


Fig. 11

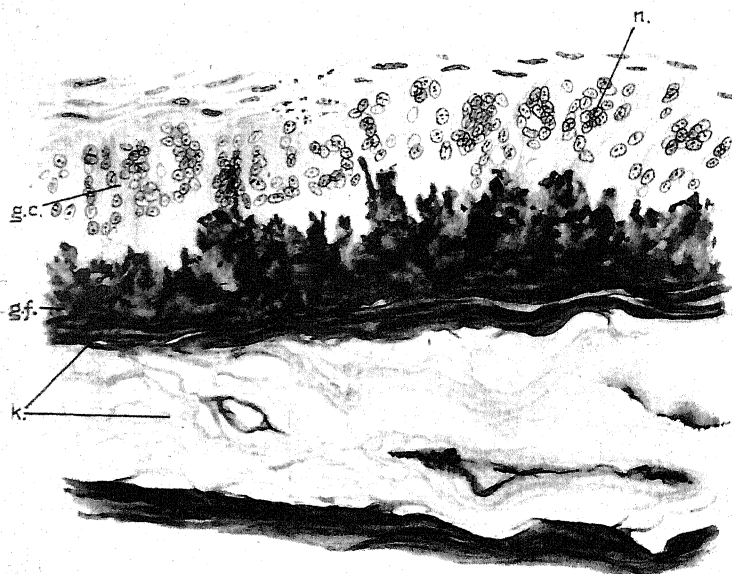


Fig 10

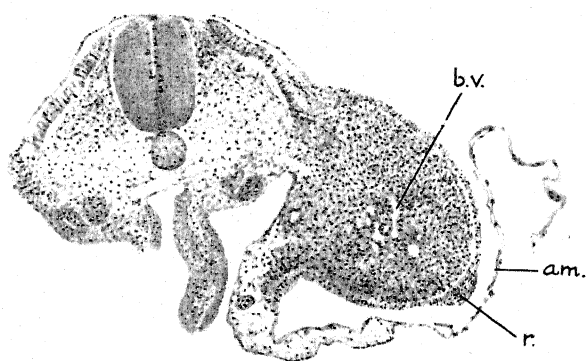


Fig. 13

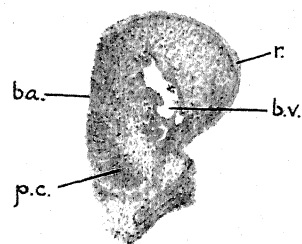


Fig. 14

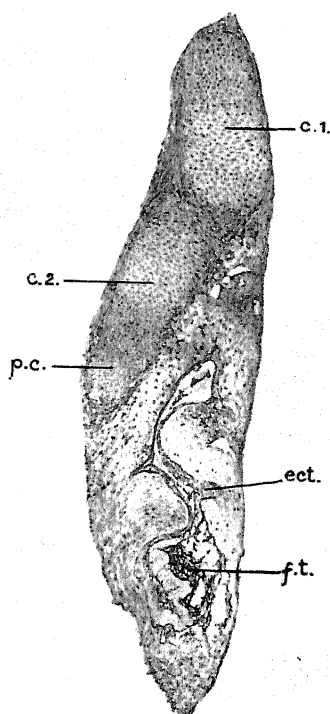


Fig. 15

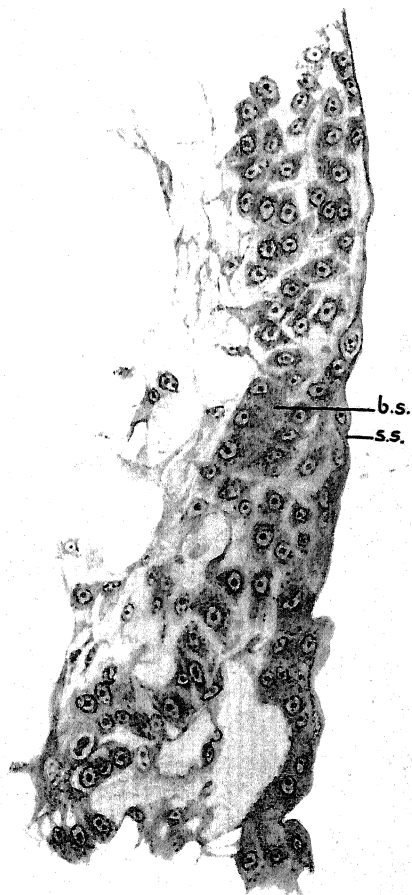


Fig. 16

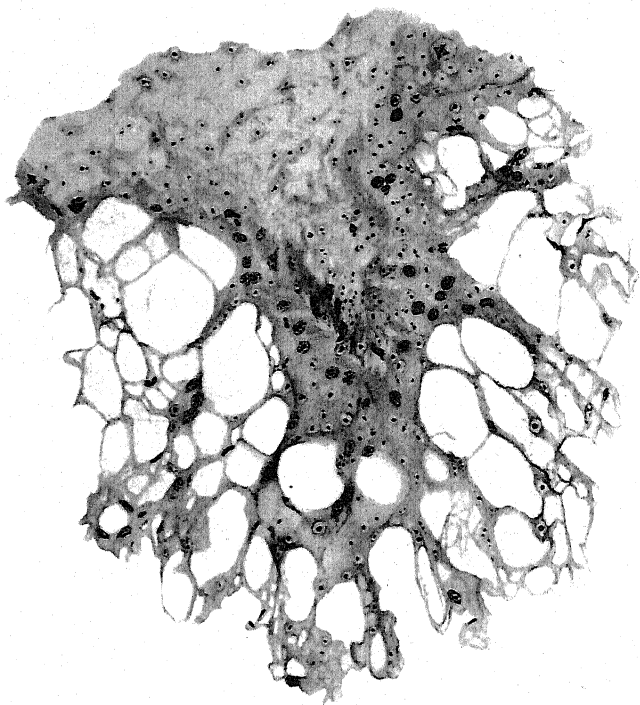


Fig. 17

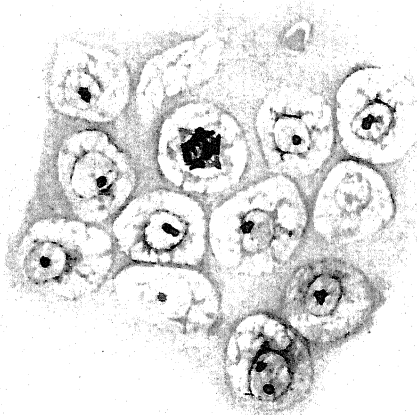


Fig. 18

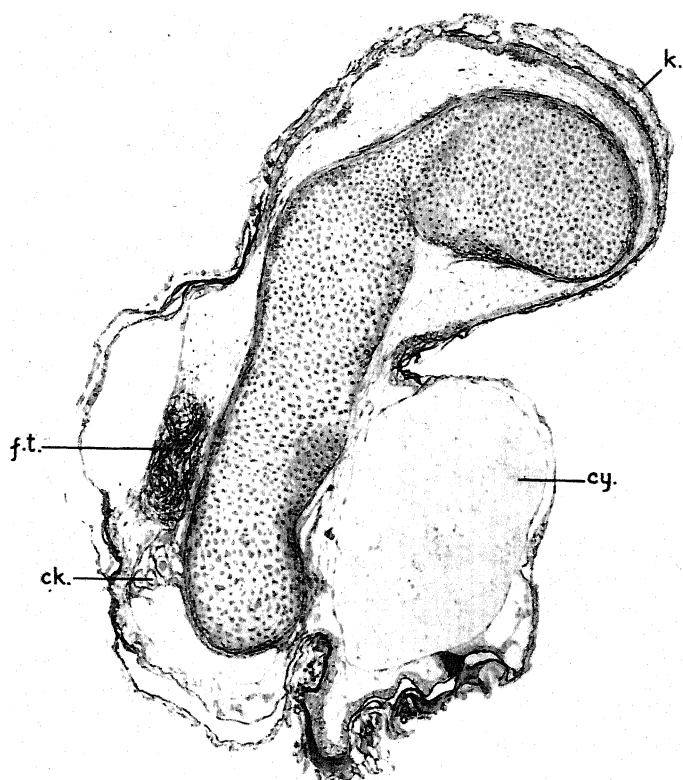


Fig. 19

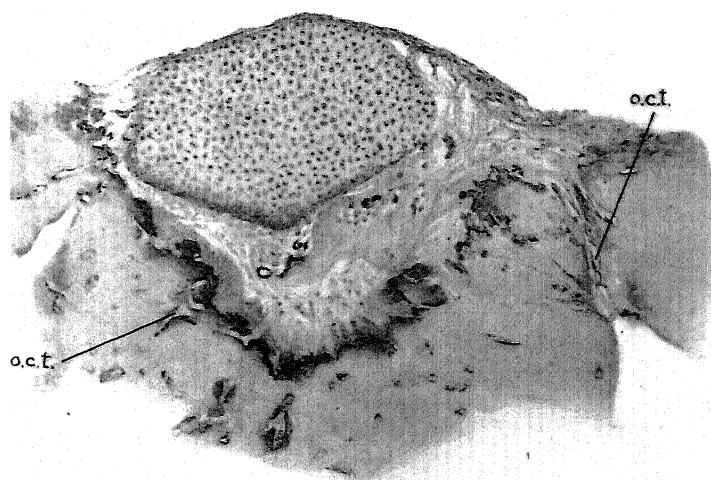


Fig. 20

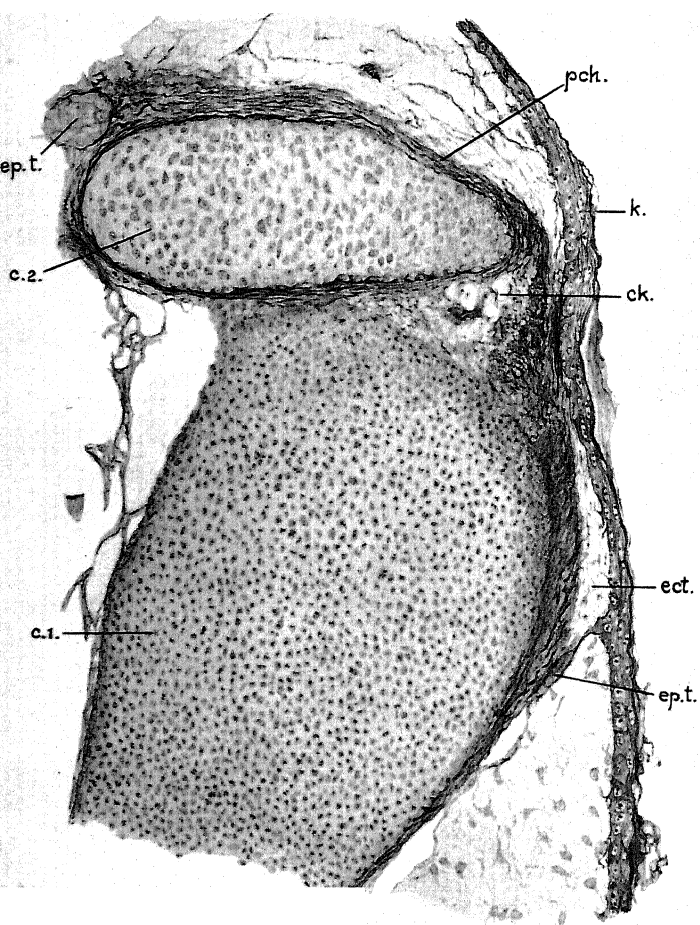


Fig. 21

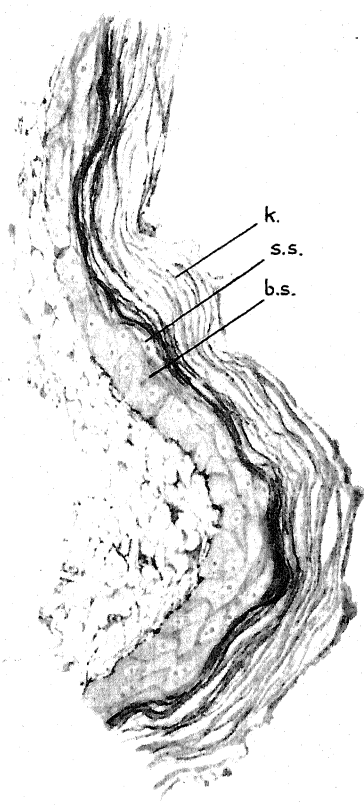


Fig. 23

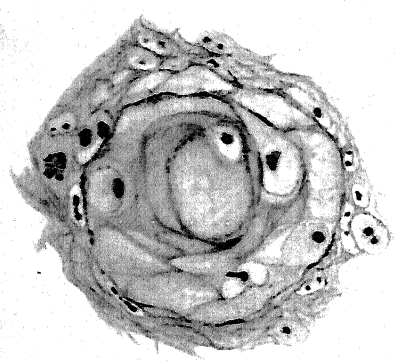


Fig. 22

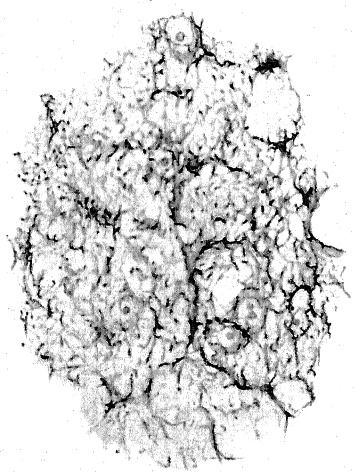


Fig. 24.



Fig. 25

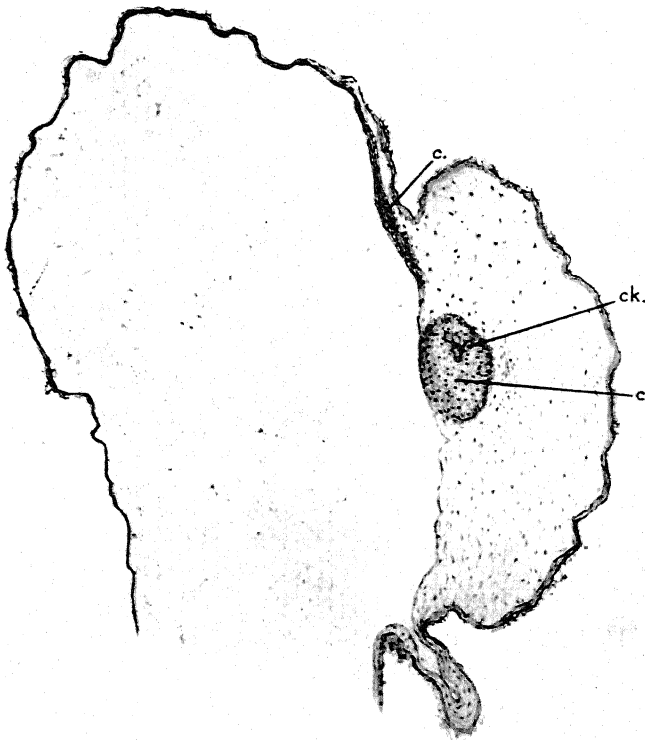


Fig. 26

together in the early telophase, gradually elongate, split longitudinally, and grow thinner, filling the daughter nucleus, when it is formed, with fine threads distributed irregularly throughout its interior, two threads being derived from each chromosome. These fine threads begin as processes from the daughter chromosomes, so that the general appearance at certain stages is that of more or less irregular elongated masses of chromatin collected together, from which fine threads, having the appearance of loops, wander through the cell. Before this appearance is lost, the nuclear membrane is formed. Not only is there a close resemblance in general appearance between this condition and that of synapsis, but I believe my observations show that "synapsis" is simply a reversal, with something added, of what happens in the somatic telophase.

The observations here described were carried out on material obtained from guinea-pigs, rats, mice, rabbits, and a monkey (*Cercopithecus Sp.?*). The changes taking place in the cells of these different animals are very similar, and I have taken those of the guinea-pig for illustration. While no pretence is made to show all the details of differences found in the various animals used, I have given some illustrations also from all of them, more particularly when they differ most from similar stages in the guinea-pig.

As the object of my observations was the elucidation of the process of reduction in the number of chromosomes, and their distribution, I have carried them no further than the telophase of the 1st Meiotic division. In dealing with the Meiotic process in Triton (Walker, 1925) my observations began with the telophase of the somatic division. I follow the same plan here. I adhere to the same nomenclature, taken from Miss Digby (1919). A *thread* is the longitudinal half of an entire univalent spireme or chromosome; a *filament* is an entire univalent spireme or chromosome produced by the lateral association of two threads.

The Somatic Division.

The longitudinal split at the earliest stage of the telophase in the daughter chromosomes in the cells of the mammals investigated is not so marked and striking as is the case with Triton, partly on account of their much smaller size and more dense packing together. This splitting may, however, often be observed at the ends of the chromosomes (Plate 14, fig. 1). At a very early stage, before the nuclear membrane is formed, fine processes may be observed proceeding from the chromatin mass formed by the grouped daughter chromosomes. Very often it is easily seen that the ends of these processes are double and enlarged. From these enlarged double ends, two fine threads

may be traced joining to what is apparently the main body of a daughter chromosome. These enlarged ends are more marked in the rat and mouse than in the guinea-pig (figs. 2-5). Sometimes a filament has elongated and extended a considerable distance from a daughter chromosome before the split has begun (fig. 4). This process goes on, the chromosome mass becoming more distributed and the arrangement losing its appearance of polarity. After the nuclear membrane is fully formed it contains a number of not very clearly-defined chromatin masses, which apparently represent that part of daughter chromosomes which has not yet completely stretched out and split into threads. A longitudinal split may often be seen in these (fig. 7).

In the tubule of the mammalian testis the cells of the different generations and all stages of these generations are frequently mixed up in an apparently inextricable confusion. I felt that statements based upon observations made only upon material in which Somatic and Meiotic divisions might be lying in close proximity, with little but the appearance of the individual cell to indicate what it was precisely, might not be very convincing, so I procured the testes of the embryo guinea-pig and of guinea-pigs up to the age of 14 days. At about the age of 14 days I found numerous somatic divisions among the early spermatogonia. Here there was no risk of confusing cells undergoing somatic with those preparing for the 1st Meiotic division. Excepting that these spermatogonia are far larger than the spermatocytes entering upon the Meiotic phase in the adult tubule, the process during the telophase is similar (fig. 6).

After the stage described in fig. 7 the apparent remnants of the daughter chromosomes disappear, and eventually the nucleus is filled by fine threads irregularly distributed, any parallel arrangement being lost (figs. 8-10). Scattered amongst this fine network are a few irregular masses of chromatin (fig. 10). This stage I regard as the end of the telophase and the beginning of the prophase. The single threads are apparently longitudinal halves of whole chromosomes, as is the case in Triton, discussed previously in much greater detail (Walker, 1925).

At this stage (fig. 10) the whole nuclear substance appears to be somewhat chromatic. This is followed by a thickening of the threads and growth of the cell. As the threads thicken they may be seen to approximate in pairs, and at the same time the nuclear substance becomes clearer (fig. 11). It may often be seen that the ends of the pairs of threads are thickened and are apparently sometimes attached to the nuclear membrane. This pairing goes on (figs. 12-14) until the nucleus is filled with filaments made up of paired threads (fig. 15). The spireme, however, is not continuous, though, as the filaments

seem frequently to follow each other in more or less continuous curves, it has somewhat the appearance of continuity. The adherence of the enlarged ends of the filaments to the nuclear membrane and the discontinuity of the spireme has been noted in other organisms (Janssens, 1925). During this period, growth of the cell has continued.

The individual chromosomes separate from each other, the nuclear membrane disappears, and the cell passes through the stages of metaphase and anaphase. I regard the longitudinal fission of the chromosomes simply as a consummation of the split which took place in the filaments in the preceding telophase (figs. 16-18).

The 1st Meiotic Division.

The telophase of the preceding division appears, according to my observations, to be so inextricably associated with the 1st Meiotic division that I have included it in the account of the latter for the sake of greater clarity.

The telophase of the last somatic division resembles that of the preceding division. The cells are, on the average, a little larger to begin with and the threads thicker. The daughter chromosomes begin to elongate and split at an earlier stage (fig. 19). The double enlargements at the ends of the filaments are more marked, as also is the frequency of their adherence to the nuclear membrane (figs. 20 and 21). Again this is more accentuated in the mouse and rat than in the guinea-pig. The same chromatic appearance of the nucleus is observed when the prophase is approached, and the same growth of the cell. This stage, the end of the last somatic telophase, is, I think, a short one, as cells in it are not numerous in any sections I have examined.

As the prophase begins the threads grow thicker and more distinct, and a few may be seen to approximate for short lengths (Plate 15, figs. 26 and 27). This continues, and eventually the threads are joined laterally in pairs throughout their length, or nearly so. While this lateral approximation is going on a certain polarity gradually appears in the nucleus.

A very striking feature, and one which appears very distinctly at this period, is that the filaments formed by the approximation of the threads, where whole ones can be traced, are discrete and have masses of chromatin at their ends which are adherent to the nuclear membrane. With regard to this point I would again refer to the observations of Janssens (1925), who observed somewhat similar phenomena in insects. I regard these discrete filaments as representing somatic chromosomes (figs. 31, 35 and 36).

The appearance of polarity is produced by the gradual migration of the

enlarged ends of the filaments to one area of the nuclear membrane (fig. 30). In time they all become crowded together there, and the threads are joined into complete filaments except at a few points. It may be seen at this period that the ends of the filaments are becoming arranged parallel to each other (figs. 32 and 33). Eventually the crowding together at the particular area of the nucleus becomes so dense that it is difficult or impossible to make out individual filaments at this spot. The appearance presents densely packed masses of chromatin, often elongated and curved, from which proceed loops. Sometimes these loops end at a distant part of the nuclear membrane in an enlarged end, but more often both ends enter into the chromatin mass (fig. 34).

At this period there begins a stage of very rapid growth. This contraction of the filaments apparently lasts some time, or is a very slow process, as a great many cells in this condition can generally be seen in any properly preserved section of the testis. It is apparently more rapid in the mouse and rat than in the guinea-pig. While the threads forming the filaments have generally joined in almost any cell, both at this stage and a little later, it can be seen that the lateral union is incomplete at certain points in some filaments, particularly at the centre of the loop. Also the masses of chromatin at the ends of the loops are frequently double (figs. 30-33).

The contraction is followed by a gradual loosening and loss of polarity (figs. 35-37). The parallel arrangement towards the ends of the filaments noticed at the beginning of synapsis can again be seen (fig. 37), but it is more generally distributed and not confined to a particular area as before (figs. 32-36). The filaments are, in fact, approximated in pairs laterally,* just as the semivalent threads approximated to form univalent filaments. As the approximation proceeds, the filaments appear gradually to become more diffuse in structure and any polarity in the arrangement of the bivalent loops thus formed is absent or very slight (Plates 15-16, figs. 38-43). At the same time the nuclear substance becomes more chromatic. The enlargement of the ends of the filaments continues to exist, and as these ends sometimes appear to remain double, the approximated ends of two filaments may occasionally have a quadruple appearance (figs. 41 and 43).

A stage is now reached when the paired filaments which are rather loose in structure, but not very intimately joined, begin to condense and become clearly defined. At the same time the chromatic appearance of the nucleus gradually

* This interpretation was facilitated by what I had already observed in Triton at the corresponding stage (Walker 1925) where the sequence of events is far more easily seen.

disappears (figs. 44-46). While the filaments individually become more and more dense in structure, their association in pairs becomes looser, and though the ends of the pairs remain adherent to each other the filaments separate gradually until they are joined in pairs by their ends only. At the same time that the filaments are becoming denser and contracting, the ends lose any sign of the quadruple formation which might be traced before. Except on very rare occasions all sign of the double structure of the filaments is lost. I have seen a few cells in these later stages where the curve of a filament showed the two threads of which it was formed, but I think that these cases are exceptional in the animals dealt with. On the other hand the double masses of chromatin joining the ends of the filaments are larger than at any previous period, and in the later stages the double filament loops appear to contract upon them (figs. 44-47). As this goes on they contract in length, and finally emerge as discrete masses of chromatin of various shapes, arranged irregularly at the periphery of the nucleus. These are the bivalent chromosomes of the 1st Meiotic division (figs. 48-50).

In the guinea-pig the chromosomes at the metaphase and anaphase of the 1st Meiotic division are not well defined and are grouped very closely together. In the rat and mouse they are more sharply defined and not so tightly packed (figs. 51 and 52).

The longitudinal splitting of the daughter chromosomes of the 1st Meiotic division appears very early in the telophase (fig. 53), and the sequence of events is similar to that of the two preceding telophases. The cells are much smaller (figs. 54-57). I do not think there is any material difference of opinion as to the longitudinal splitting of the chromosomes in the 2nd Meiotic division.

I believe that as the longitudinal division of the daughter chromosomes during the telophase is the precursor of and is consummated in the anaphase of the following somatic division; so the longitudinal division of the daughter chromosomes into semivalent threads in the telophase of the last somatic division is the precursor of and is consummated only at the 2nd Meiotic division, constituting a unique phenomenon which provides for the distribution of whole chromosomes instead of longitudinal halves, during, and as the result of, the 1st Meiotic division.

Apart from the longitudinal split of the daughter chromosomes, the separation of each into two semivalent threads, and the subsequent reunion of these threads into univalent filaments, my observations agree in the main with those of many other investigators. Bouin (1925) describes the daughter chromosomes as lengthening (*s'allongent*) until the nucleus is filled by a mass of delicate

filaments which are not orientated. He describes the ends as remaining thicker and being bent inwards producing orientation (*bouquet orienté*). He describes a lateral conjugation of the threads and a subsequent separation. Janssens (1925) in a very exhaustive contribution on this process in insects (also touching upon some other organisms), does not deal with the telophase at great length. He does not observe a longitudinal division of the daughter chromosomes, but some of his figures appear to me as though they might possibly bear that interpretation.

Many authors have described the filaments in many different organisms which I believe to be the univalent filaments formed by the lateral reunion of the semivalent threads, as joining laterally, shortening and thickening to form the bivalent chromosomes of the 1st Meiotic division. (Janssens, 1901; Champy, 1913; Lérat, 1905; Van Molle, 1907; Agar, 1911.)

Miss Digby (1919) described the separation of the chromosomes into two semivalent threads and their subsequent reunion in *Osmunda*, the general outline of events being very similar to those described here. Dehorne (1911) described a longitudinal separation, but describes quite a different series of changes as following. I dealt with these observations at greater length on a previous occasion (Walker, 1925), and have seen no further publications since having a direct bearing upon the subject.

Summary.

Briefly, I believe that what happens in the Meiotic phase in the animals dealt with is as follows :—

The daughter chromosomes elongate and divide longitudinally, filling the nucleus with irregularly distributed semivalent threads. During this process of unravelling of the chromosomes into semivalent threads, an appearance very similar to that seen during synapsis is evident. These threads rejoin laterally in pairs forming univalent filaments. These filaments join laterally in pairs. They again separate, excepting at their ends which remain joined until the 1st Meiotic division takes place. The longitudinal split of the filament into semivalent threads reappears at the telophase, and is consummated at the 2nd Meiotic division.

Notes.

1. As the phenomenon of the separation of the daughter chromosomes or filaments into two semivalent threads appears to be of importance, both in the somatic and Meiotic divisions, I would suggest the term "exileisis" for the

process, derived from *ἐξείλησις*, a disentangling. I owe the suggestion of the term to Sir Ray Lankester.

2. Some appearances will be observed in certain figures (*e.g.*, figs. 42, 51, 52, 53, 54) which suggest the possible presence of accessory chromosomes. I have not followed up the investigation of these.

3. In the early spermatogonia of the embryo shortly before birth, and of the very young guinea-pig, an appearance which suggests amitosis is to be observed. I have not investigated this appearance in detail, but from what I have seen I am inclined to believe that, as I concluded was the case with regard to a similar appearance in the testis of Triton (Walker, 1925), amitotic division does not take place. As in Triton, pluripolar division figures are found among the early spermatogonia.

4. The Telophase of the Somatic division in Triton bears a very distinct resemblance to the Synaptic figures in Mammalia and other organisms where it occurs. There is no Synapsis in Triton. The figures in my paper on Triton (Walker, 1925) illustrate this resemblance, though I did not appreciate the fact when they were published.

BIBLIOGRAPHY.

- Agar, 'Quart. Journ. Micro. Sci.,' vol. 57 (1911).
 Blackman, 'Biol. Bull.,' vol. 5, No. 4 (1903).
 Bouin, P., 'La Cellule,' vol. 35, p. 373 (1925).
 Champy, 'Arch. de Zool. Expér.,' vol. 52 (1913).
 Dehorne, 'Arch. de Zool. Expér.,' vol. 9, p. 1-175 (1911).
 Digby, 'Annals of Botany,' vol. 33, No. 103 (1919).
 Janssens, 'La Cellule,' vol. 19, f.1(1901); *ibid.*, vol. 22, f. 2 (1905); *ibid.*, vol. 25, f. 2 (1909);
ibid., vol. 34, f. 1 (1925).
 Lérat, 'La Cellule,' vol. 22 (1905).
 Van Molle, 'La Cellule,' vol. 24 (1907).
 Walker, 'Roy. Soc. Proc.,' B, vol. 98 (1925).
 Flemming, 'Arch. f. Mikr. Anat.,' vol. 25 (1911).

DESCRIPTION OF PLATES 14-16.

All the Figures except where otherwise stated are from the guinea-pig.

The magnification of the figures varies. The line beside each represents 10 μ . This scale was taken with a stage micrometer and the camera lucida at the same time that each cell was drawn.

PLATE 14.

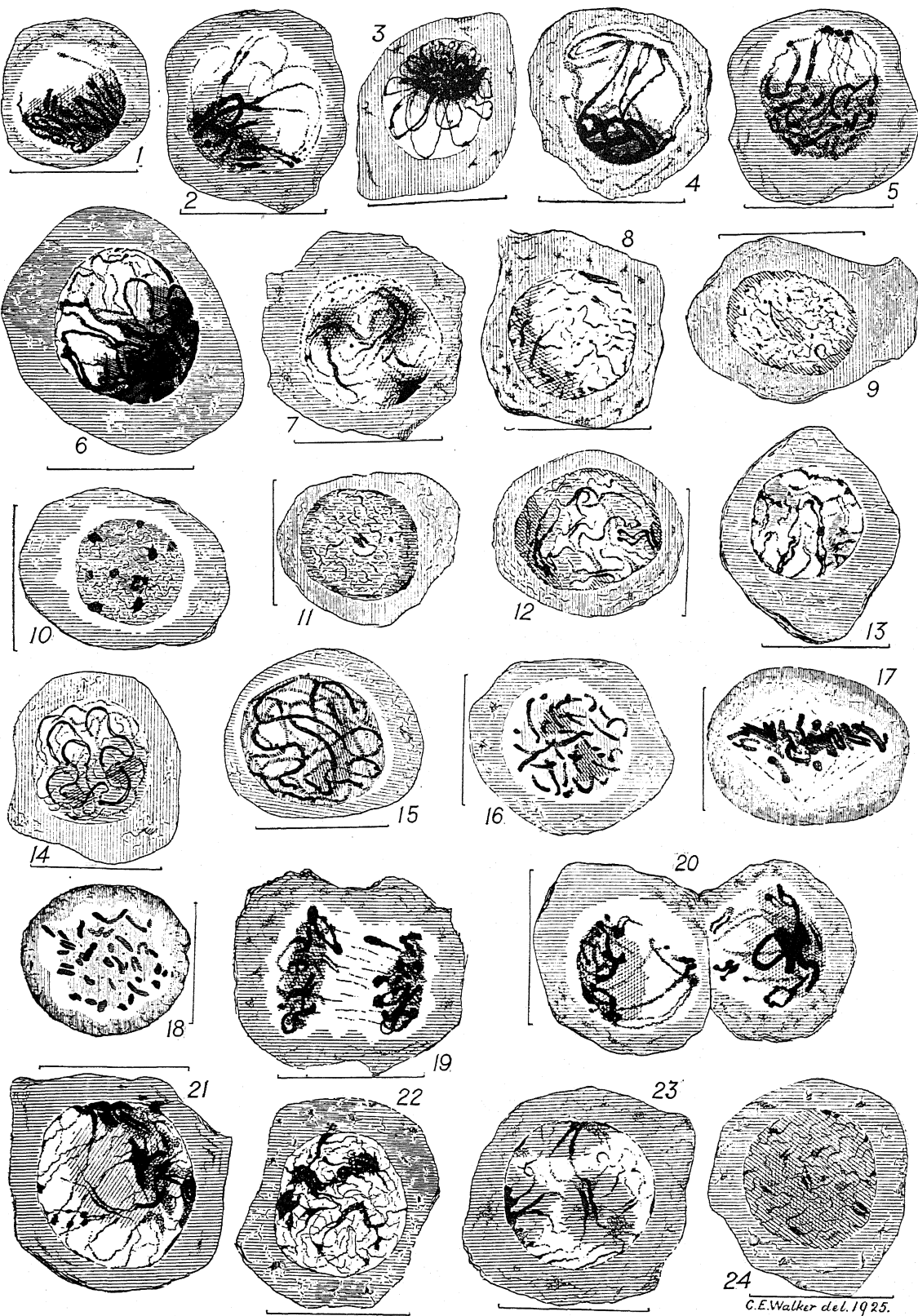
- Fig. 1.—Early telophase. Somatic division.
- Fig. 2.—Same later.
- Fig. 3.—Similar stage from rabbit.
- Fig. 4.—Similar stage from mouse.
- Fig. 5.—Similar stage from rat.
- Fig. 6.—Similar stage from spermatogonium of guinea-pig 14 days old.
- Fig. 7-9.—Progressive stages of telophase.
- Fig. 10.—End of telophase and beginning of prophase.
- Fig. 11.—Early prophase, somatic division.
- Figs. 12-16.—Progressive stages of same.
- Figs. 17-18.—Metaphase.
- Fig. 19.—Late anaphase.
- Figs. 20 to 24.—Progressive stages of telophase of last somatic division.

PLATE 15.

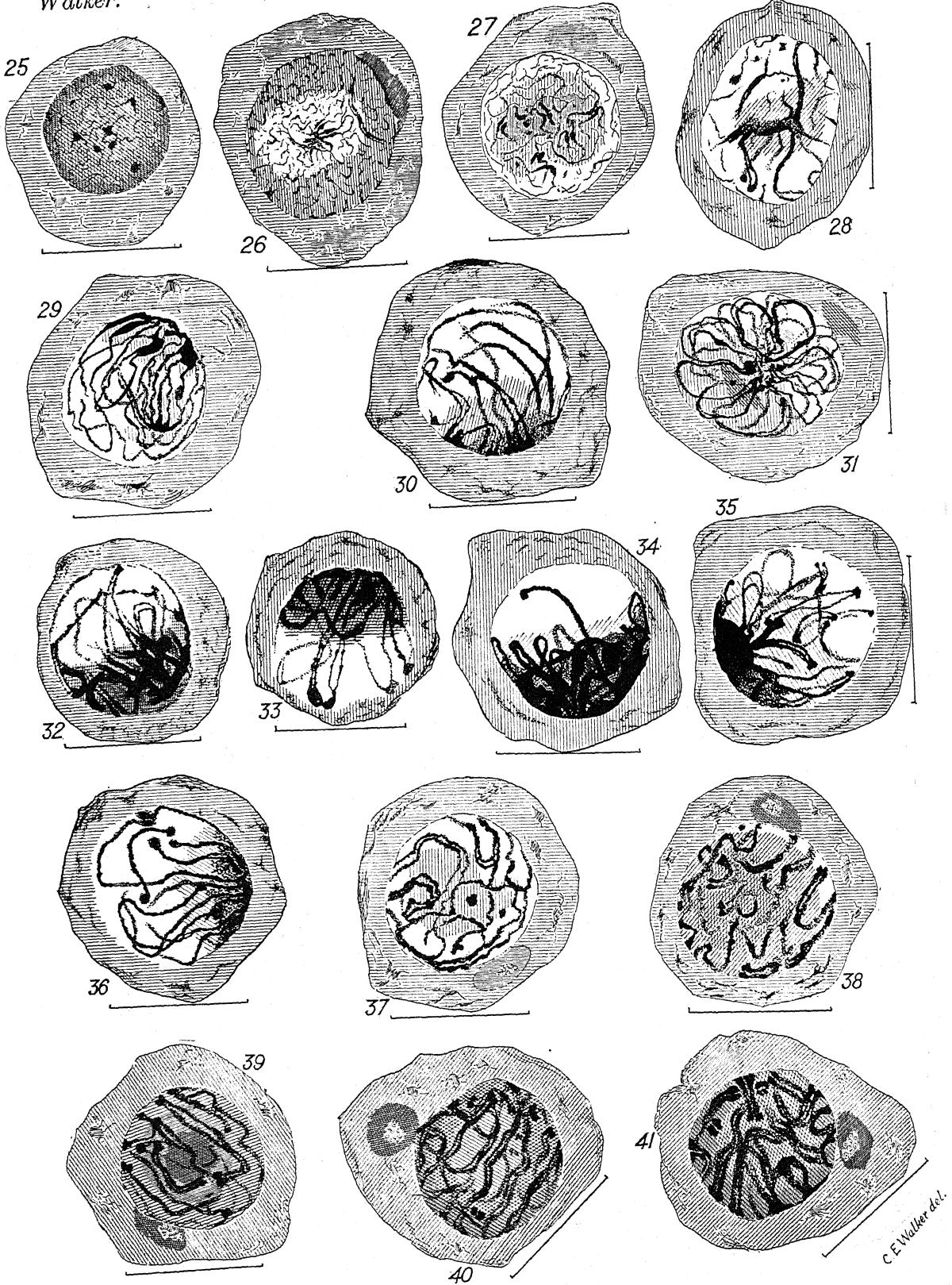
- Fig. 25.—End of telophase and beginning of prophase of 1st Meiotic division.
- Figs. 26 to 28.—Pairing of semivalent threads.
- Fig. 29.—Commencement of pairing of univalent filaments and synapsis.
- Fig. 30.—Polarity becoming evident.
- Fig. 31.—Polar view of similar stage.
- Figs. 32 to 34.—Completion of synaptic contraction. Filaments parallel in some cells.
- Figs. 35 to 37.—Gradual loss of polarity.
- Fig. 38.—Pairs of filaments becoming more diffuse in structure. Nuclear substance becoming chromatic.
- Figs. 40 to 43.—Progressive diffuseness of paired filaments and chromatisation of nuclear substance.

PLATE 16.

- Fig. 42.—From rat.
 - Figs. 44 and 45.—Condensation of filaments which begin to separate. Clearing of nuclear substance.
 - Fig. 46.—Similar stage from monkey (*Cercopithecus* Sp ?).
 - Fig. 47.—Further separation and condensation of filaments. Ends of paired filaments remain attached.
 - Figs. 48 to 50.—Formation of chromosomes.
 - Fig. 51.—Metaphase of 1st Meiotic division in rat.
 - Fig. 52.—Ditto in guinea-pig.
 - Figs. 53 to 57.—Telophase of 1st Meiotic division.
-

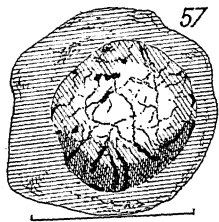
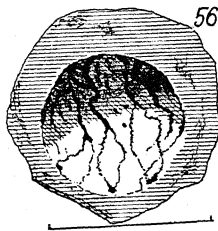
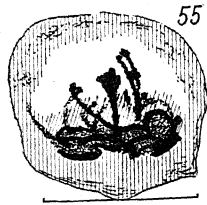
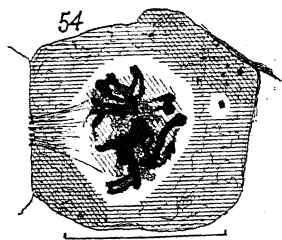
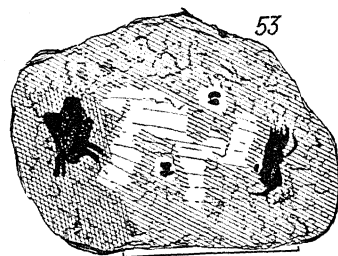
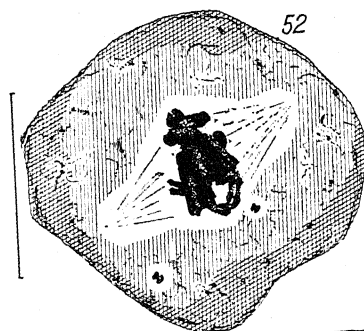
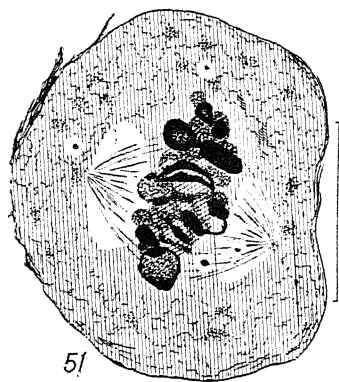
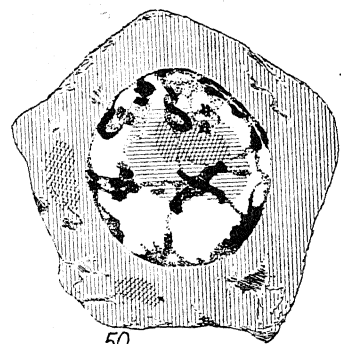
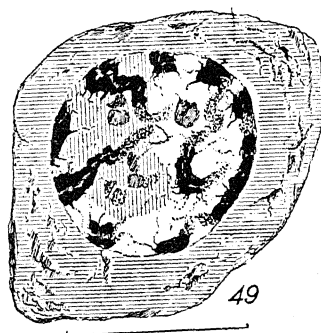
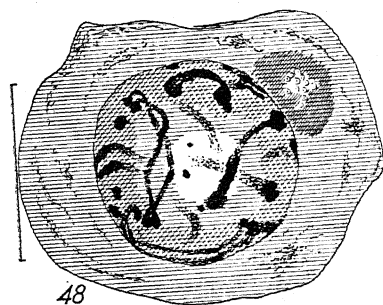
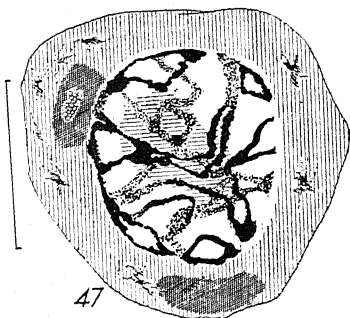
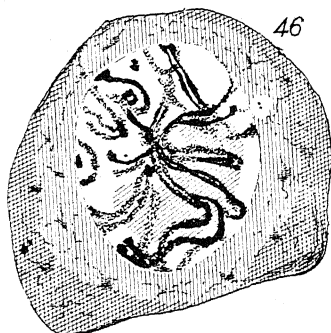
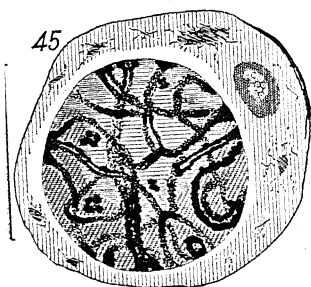
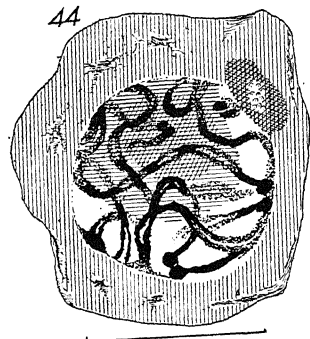
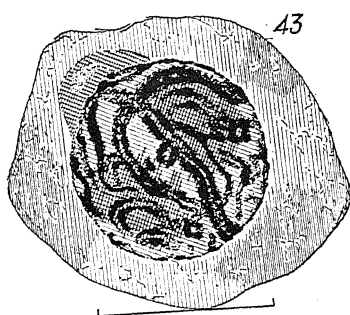
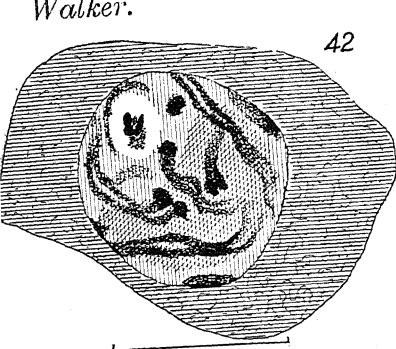


Walker.



C. E. Walker del.

Walker.



C.E. Walker del.

The Effect of Insulin on the Dextrose Consumption of Perfused Skeletal Muscle.

By CHARLES H. BEST.*

(Communicated by Dr. H. H. Dale, Sec. R.S.—Received December 23, 1925.)

(From the National Institute for Medical Research, London.)

The experiments of Hepburn and Latchford (1), which have been confirmed by Burn and Dale (2), show that insulin accelerates the rate of disappearance of dextrose from the fluid used to perfuse the isolated mammalian heart. Burn and Dale also demonstrated that insulin greatly increases the rate of disappearance of dextrose from the circulating blood of the decapitated and eviscerated cat. Cori, Cori and Goltz (3), working on rabbits, and Lawrence (4) and Pemberton and Cunningham (5), from clinical studies, have reported that insulin increases the loss of sugar from the blood during its passage through a limb. Frank, Nothman and Wagner (6) have obtained similar results by analyses of blood samples drawn simultaneously from the femoral artery and vein, after the injection of insulin into the femoral artery. Macleod (7) states that in experiments in his laboratory, no increased discrepancy between the dextrose content of the arterial and venous blood was observed after the administration of insulin in normal or diabetic animals.

Attempts to prove that insulin causes an increased disappearance of sugar from the fluid perfused through the isolated limbs of laboratory animals have been made by Macleod and his collaborators (7) and Staub (8). Macleod states that his experiments were unsatisfactory because of cedema of the muscles or the development of marked resistance to the perfusion. Staub has reported experiments in which the rate of sugar disappearance, before and after the addition of insulin, from the defibrinated blood used to perfuse the hind limbs of the dog, are recorded. In some of Staub's experiments insulin appeared definitely to accelerate the sugar disappearance. Because of the very rapid disappearance of sugar from the blood before the addition of insulin, however, it is difficult to demonstrate convincingly, by this type of experiment, that the rate of disappearance is really accelerated by insulin.

Since an efficient perfusion apparatus, by which the perfusion fluid is circulated continuously was available in this laboratory, it was thought worth while to attempt to obtain more convincing evidence of the effect of insulin on the

* Fellow of the International Health Board of the Rockefeller Foundation.

disappearance of sugar from fluid perfused through a skeletal muscle preparation. In the experiments reported in this paper defibrinated blood was perfused through the vessels of the hind limbs of the cat.

Methods.

The sugar concentration of the blood was prevented from falling rapidly in the preliminary period by the addition of dextrose at a known rate. When insulin was added to the blood, the rate of this artificial addition of dextrose was usually increased, so as to mitigate the fall produced by insulin. Full particulars of the perfusion apparatus used in these experiments are given in a recent publication by Burn and Dale (9). The following procedure by which the tissue was set up for perfusion is similar to that described by these investigators. The anaesthetised cat was bled from the aorta. About 50 c.c. of saline were run in through the jugular vein during the bleeding. After the death of the animal mass ligatures were applied at the level of the upper lumbar vertebrae. The hind quarters were then separated from the rest of the body by a transection just above the mass ligatures, and the spinal canal was firmly plugged with plasticine. Cannulae were placed in the aorta and the vena cava, and connections with the arterial and venous blood tubes of the perfusion apparatus were made.

In many of the experiments it was necessary to use the blood from two cats, since the perfusion apparatus was constructed for a volume of 225 c.c. The blood was defibrinated by whipping and was then filtered through cotton-wool. The blood flow through the limbs was kept as nearly constant as possible throughout the experiment. The burette of the slow infusion apparatus described by Burn and Dale (2) was connected by rubber tubing with a small glass tube, which was fitted in a hole bored in the top of the oxygenating chamber, so that 4 per cent. dextrose was added at a known rate to the blood and completely mixed with it during oxygenation. In all the experiments the infusion of dextrose was started as soon as the perfusion was properly under way. Blood samples were taken every 20 minutes from the reservoir which receives the blood from the pump. As much blood as possible was kept in the reservoir during the experiment, so that these samples should be fairly representative of the whole volume of blood in circulation. The dextrose determinations were made by the Shaffer-Hartmann method. Insulin was added to the blood by delivering the required quantity, dissolved in 0.25 to 0.5 c.c. of saline, into the funnel which receives the blood from the venous cannula.

The values for the sugar disappearance were calculated from the sugar-infusion figures and the change in sugar concentration of the blood. In the

calculations the volume was assumed to be the same as the volume of blood originally placed in the apparatus. During the experiment the blood was concentrated somewhat by evaporation of water during oxygenation and was diluted slightly by the fluid in which the sugar was added. The discrepancy between the volume of blood recovered from the apparatus, *plus* the amount used for analysis, *plus* or *minus* the gain or loss of weight of the perfused tissue on the one hand, and the amount of blood originally taken *plus* the volume of sugar solution added on the other hand, is accounted for by evaporation. This was tested in some experiments by hæmoglobin estimations made at the beginning and end of the experiment, the loss of water calculated from these accounting satisfactorily for the missing volume.

Experimental Results.

The essential details of the experiments are recorded on the graphs. Only very brief protocols are therefore submitted.

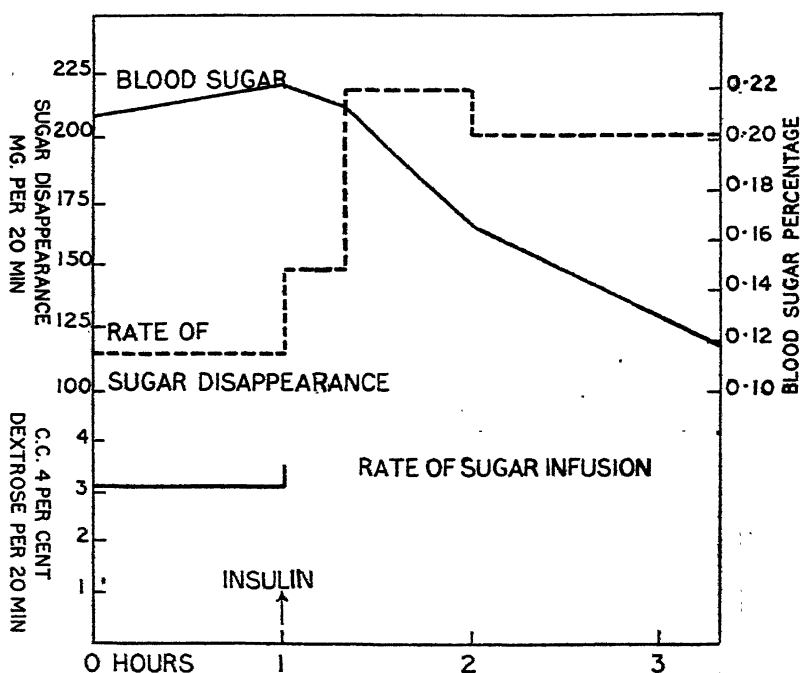


FIG. 1.

Experiment 1 (fig. 1).—Weight of tissue perfused was 1248 grams. Volume of blood used in perfusion was 220 c.c. Blood samples were taken every

20 minutes. The blood-sugar values were 0.210 per cent., 0.212 per cent., 0.223 per cent., 0.223 per cent. (insulin 10 units), 0.220 per cent., 0.188 per cent., 0.168 per cent., 0.161 per cent., 0.146 per cent., 0.132 per cent., 0.119 per cent. The sugar infusion, as shown in fig. 1, was increased when insulin was added. At the end of the experiment 125 c.c. of blood were recovered from the apparatus. The perfused tissue increased in weight by 52 grams. 34 c.c. of blood had been taken during the experiment for analysis. 41 c.c. of 4 per cent. sugar solution were added to the blood during the experiment. Rate of blood flow before insulin, 2080 c.c. per hour; rate of blood-flow in first hour after insulin, 2307 c.c. per hour. Temperature was 37-38° C.

Calculation shows that in the hour before insulin 376 milligrams of sugar disappeared, while in the first hour after insulin the disappearance was 643 milligrams.

Experiment 2 (fig. 2).—Weight of tissue perfused was 652 grams. The volume of the blood used was 270 c.c. The sugar in the blood was estimated every 20 minutes. The values were as follows: at the beginning, 0.178 per

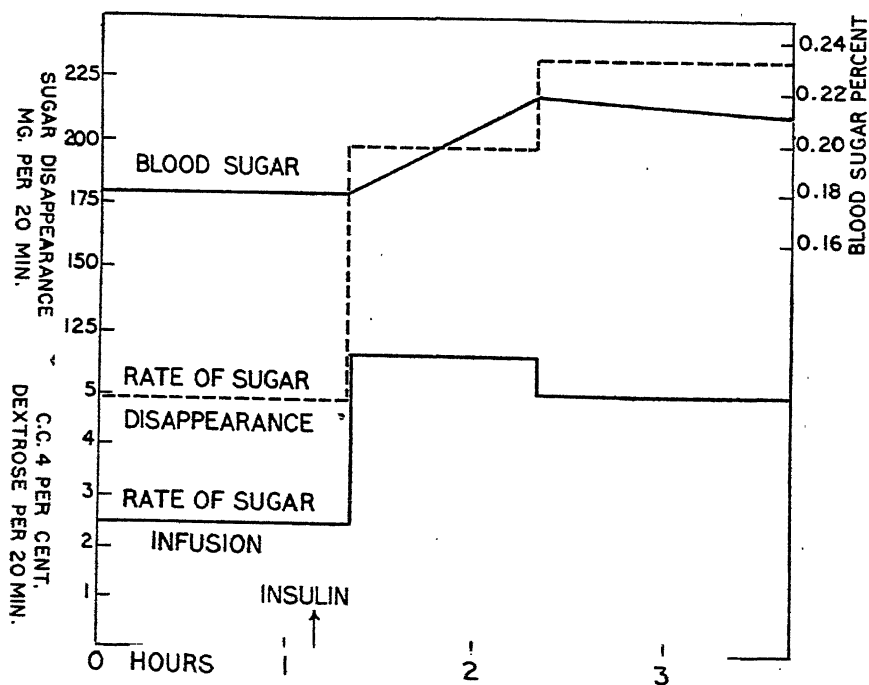


FIG. 2.

cent., 0.174 per cent., 0.183 per cent., 0.178 per cent. (insulin 15 units), 0.178 per cent., 0.196 per cent., 0.221 per cent., 0.219 per cent., 0.229 per cent., 0.224 per cent., 0.197 per cent., 0.210 per cent. Volume of blood recovered at the end of the experiment was 230 c.c. Samples accounted for 26 c.c. Weight of tissue at end of experiment was 642 grams. 47.6 c.c. of sugar solution were added. Rate of blood flow before insulin, 2352 c.c. per hour; rate of blood-flow in first hour after insulin, 2122 c.c. per hour. Temperature, 37–38° C.

In the hour before insulin was added to the blood 324 mg. of sugar disappeared. After insulin 620 mg. per hour disappeared.

*Experiment 3 (fig. 3).—*Weight of tissue perfused was 782 grams. 260 c.c. of defibrinated blood were used in the perfusion. The sugar values, determined

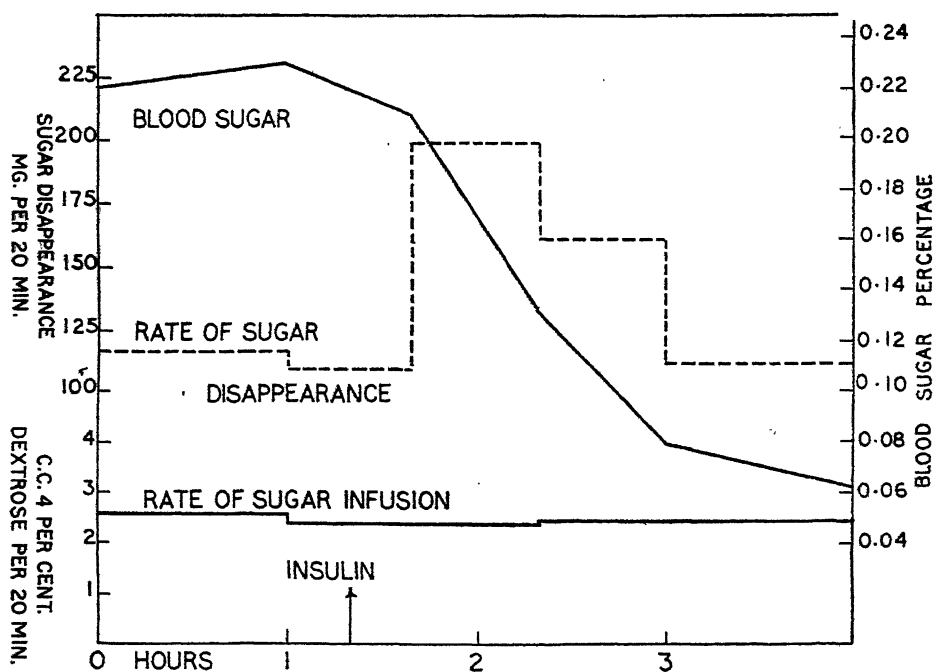


FIG. 3.

at 20-minute intervals throughout the experiment, were 0.221 per cent., 0.238 per cent., 0.224 per cent., 0.232 per cent., 0.215 per cent. (insulin 15 units), 0.210 per cent., 0.173 per cent., 0.133 per cent., 0.118 per cent., 0.080 per cent., 0.070 per cent., 0.060 per cent., 0.062 per cent. 150 c.c. of blood

were recovered from the apparatus (samples accounted for 36 c.c.). 29 c.c. of 4 per cent. sugar solution were infused. Weight of tissue at end of experiment was 835 grams. Rate of blood flow before insulin, 2396 c.c. per hour; rate of blood-flow in the first hour after insulin, 2374 c.c. per hour. .

Sugar disappearance before insulin was 325.6 mg. per hour. In the first hour after insulin the disappearance was 530 mg.

Experiment 4.—Weight of tissue perfused was 906 grams. Volume of blood used in the perfusion was 250 c.c. Sugar was infused at the rate of 4.8 mg. per minute. The sugar concentration of the blood remained steady at 0.20 per cent. for the first three 20-minute intervals. The sugar infusion was then stopped and the tissue was eliminated from the circulation. The sugar determinations for the next four 20-minute periods were 0.20 per cent., 0.20 per cent., 0.20 per cent., 0.19 per cent. Fifteen units of insulin were then added to the circulating blood. The next three sugar values were 0.19 per cent., 0.18 per cent., 0.18 per cent. Blood recovered from the apparatus was 160 c.c. Weight of tissue at end of experiment was 932 grams. 8.5 c.c. of sugar solution were infused. 28 c.c. of blood were used for sampling.

This experiment shows that there is little or no disappearance of sugar from the blood, either before or after the addition of insulin, when the tissue is eliminated from the circulation.

Experiment 5 (fig. 4).—Weight of tissue perfused was 1212 grams. Volume of blood was 220 c.c. The blood sugar was determined at twenty minute intervals. The first three values were 0.220 per cent., 0.210 per cent., 0.204 per cent. 15 units of insulin were then added. The next three sugar values were 0.169 per cent., 0.124 per cent., 0.083 per cent. The sugar infusion was then stopped, and the tissue was eliminated from the circulation. The next four sugar values were 0.092 per cent., 0.087 per cent., 0.080 per cent., 0.076 per cent. The slight rise in sugar concentration was probably due to the fact that the last bit of infused sugar was not mixed with the blood at the time the 0.083 per cent. sample was taken. Rate of blood flow before insulin 2280 c.c. per hour. Rate of blood flow after insulin 2800 c.c. per hour. One hour and 40 minutes after the beginning of the experiment the perfusion through the limbs and the addition of sugar were stopped. The warm blood was pumped through the apparatus during the remainder of the experiment. Temperature throughout experiment was 37–38° C.

This experiment is the same as the preceding, except that insulin was added before the tissue was excluded from the circulation. The results are the same as in Experiment 4.

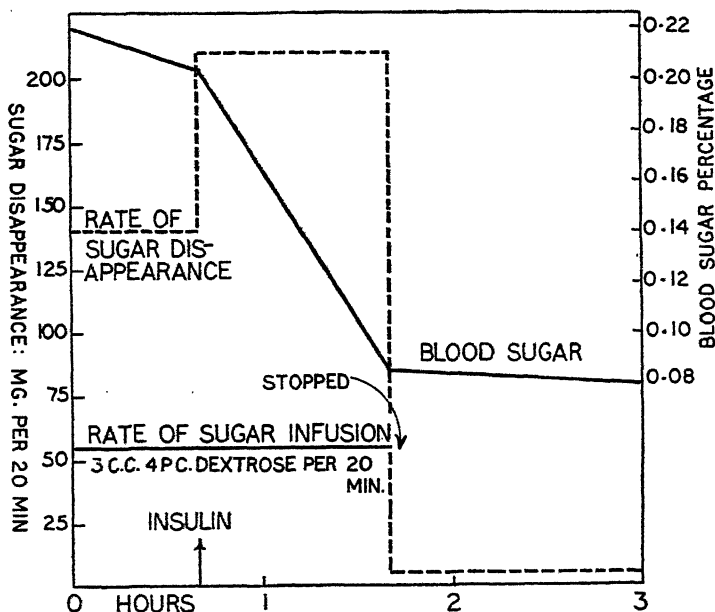


FIG. 4.

Discussion.

Burn and Dale used a preparation consisting of heart, lungs, skeleton, skin and muscles. They showed that removal of the skin did not affect the result. In the experiments here described, the heart and lungs have been replaced by a mechanical system, so that it is safe to attribute the effects observed chiefly, if not entirely, to the metabolic activity of the skeletal muscles. Under these conditions, the rate of sugar disappearance from the blood is very rapid, even before the addition of insulin. It is much more rapid, per gram of muscle, than that from the blood of the decapitated and eviscerated cat before insulin is administered. The extent to which the liver, although cut off from direct circulation, contributes sugar by diffusion to the eviscerated preparation is not known. Slightly more sugar, therefore, than would appear from the determinations may disappear from the blood of Burn and Dale's preparation. On the other hand, the sugar disappearance before insulin in the experiments reported in this paper might be slower if loss of CO_2 from the blood were prevented. Glycolysis by the blood itself, however, is insignificant in the

defibrinated blood which has passed through cotton-wool, and plays no appreciable part in the observed disappearance of the sugar. In all the experiments carried out in this investigation, the acceleration of sugar disappearance after the addition of insulin was perfectly definite.

Summary.

Insulin greatly accelerates the rate of sugar disappearance from defibrinated blood used to perfuse the isolated limbs of the cat. The action is attributable to effect on the metabolism of the skeletal muscles.

The author gratefully acknowledges the kindly criticism and help of Dr. H. H. Dale in the planning and execution of the experiments reported in this paper.

REFERENCES.

- (1) Hepburn and Latchford, 'Amer. J. Physiol.,' vol. 62, p. 177 (1922).
 - (2) Burn and Dale, 'Jour. of Physiol.,' vol. 59, p. 164 (1924).
 - (3) Cori, Cori and Goltz, 'J. Pharm. and Exper. Ther.,' vol. 22, p. 355 (1923).
 - (4) Lawrence, R. D., 'Brit. Med. Journ.,' vol. 1, p. 516 (1924).
 - (5) Pemberton and Cunningham, 'The Lancet,' vol. 2, p. 1222 (1925).
 - (6) Frank, Nothman and Wagner, 'Klin. Wochenschr.,' vol. 3, p. 581 (1924).
 - (7) Macleod, 'Physiol. Review,' vol. 4, p. 21 (1924).
 - (8) Staub, H., 'Insulin' (Springer) (1924).
 - (9) Burn and Dale, 'Jour. of Physiol.' *In press.*
-

*Further Micro-Injection Studies on the Oxidation-Reduction
Potential of the Cell-Interior.*

By JOSEPH NEEDHAM, M.A., Ph.D., Fellow of Caius College, Cambridge, and
DOROTHY MOYLE NEEDHAM, Girton College, Cambridge, Beit Memorial
Research Fellow.

(Communicated by Prof. Sir Frederick Hopkins, F.R.S.—Received January 13,
1926.)

(From the Biochemical Laboratory, University of Cambridge.)

CONTENTS.

	PAGE
Introduction	383
Technique	384
<i>Amœba proteus</i> —	
(1) Injection of the series of <i>r</i> H indicators.....	389
(2) Injection of an <i>r</i> H indicator completely reduced.....	390
(3) Injection of <i>p</i> H and <i>r</i> H indicators under aërobic and anaërobic conditions	391
<i>Nyctotherus cordiformis</i> —	
(1) Determination of the internal <i>p</i> H and <i>r</i> H under aërobic conditions	393
(2) Determination of the internal <i>p</i> H and <i>r</i> H under anaërobic condi- tions	393
<i>Opalina ranarum</i>	394
General discussion	395
Summary	396

Introduction.

In previous communications on this subject (20, 21, 22) we described the results obtained when coloured indicators of known physico-chemical properties were injected into individual living cells. Using a modification of the micromanipulator of Chambers (4), we have worked with various unicellular protozoa and egg-cells, and have been able to draw definite conclusions as to the average hydrogen-ion concentration and the average oxidation-reduction potential of the cell interior. Our first communication dealt with the amœba, and we showed that its internal *p*H was probably in the neighbourhood of 7·6, while its internal *r*H (oxidation-reduction potential, 5) was between 17 and 19. Both values are near neutrality, so that this cell could be said to be slightly alkaline and slightly on the electronegative or reducing side of oxidation-reduction neutrality.

We next extended our investigation to several types of marine egg-cells before and after fertilisation, and during the early cleavage stages. The changes which the internal pH and rH undergo during these ontogenetic events are very small indeed, and the phylogenetic differences, for example, as between the ovum of the polychaete worm and that of the starfish are correspondingly slight. The egg-cell, then, appeared to have a pH of about 6.6 and an rH of the order of 21 or 22. It was therefore a little on the acid side of acid-base neutrality and a little on the electropositive side of oxidation-reduction neutrality, differing on both these counts from the amoeba. The amoeba, therefore, has a higher intensity of reduction than the egg-cell.

At the conclusion of our last paper we wrote:—"Mansfield Clark (6) has suggested that some micro-organisms may have an rH of slightly less than 0. We propose next to investigate the effect of anaërobiosis, in the hope that the fixing of the physiological range of rH may lead to clearer ideas as to the extent of the alteration likely to take place in the individual." The present paper is for the most part a report of our results in this matter of anaërobiosis. We have investigated the internal rH of *Amœba proteus* in atmospheres of pure oxygen and pure hydrogen, and we have determined the internal rH of *Nyctotherus cordiformis*, an anaërobic ciliate, parasitic in the frog's intestine, under both aërobic and anaërobic conditions. We also describe some further work on the injection of completely reduced indicator into *Amœba proteus* and a few experiments on *Opalina ranarum*.

Technique.

The pH indicators were those in ordinary use, and of the rH indicators it need only be said that they lie in order on the oxidation-reduction scale, so that by seeing which of them is oxidised or reduced in a given cell deductions may be drawn as to the relative position of the cell on the scale. For the sake of convenience we give them all in Table I, which shows their electrode and oxidation-reduction potentials at pH 7.0 and 50 per cent. reduction. For the concentration in which the dyes were used, and for other details of technique relating to them, reference may be made to our preceding paper (20). As regards the protein error of these rH indicators, no data are as yet available; when they are obtained, it will probably be necessary to apply corrections to the data given in this paper.

We do not find it possible to obtain pH values by this method more accurately than ± 0.1 unit pH . This is not the fault of the comparator methods we use, but is due to the difficulty of distinguishing and matching colour in the

granular cell-material. The use of a more accurate standard of comparison is accordingly superfluous. ± 0.1 unit pH corresponds to an error of 5 per cent. on the total indicator-range, and only under the very best optical conditions is it possible to attain to an error as low as 1 per cent. (for intensity changes *see* 15, for tint changes *see* 16 and 28).

Table I.

Number of reference in this paper.	Name.	At 50 per cent. reduction and pH 7.0.	
		Eh in volts.	pH (approx.).
	(Oxygen electrode)	+ 0.810	41.0
9	0-chlorophenolindophenol	+ 0.233	20.9
8	2, 6, dibromophenolindophenol	+ 0.219	20.3
7	0-cresolindophenol	+ 0.194	19.7
6	0-cresolindo 2, 6, dichlorophenol	+ 0.181	19.3
5	1-naphthol 2-sulphonic acid indophenol	+ 0.123	17.4
4	1-naphthol 2-sulphonic acid indo 2, 6, dichlorophenol	+ 0.118	17.2
3a	Methylene blue	+ 0.025	14.4
3	Indigotine tetrasulphonic acid	- 0.058	11.9
2	Indigotine trisulphonic acid	- 0.090	10.3
1	Indigotine disulphonic acid	- 0.129	9.2
	(Hydrogen electrode)	- 0.420	0.0

For the injection of the completely reduced dye the apparatus shown in fig. 4 of our first paper (I, p. 277) was used. For micro-injection in atmospheres of varying oxygen concentrations, however, or under anaërobic conditions, a new apparatus was necessary, and we availed ourselves accordingly of an idea of Barber's (2). In his original paper on micromanipulation he suggested that for the isolation of anaërobic bacteria it might be useful to bend a micropipette in the form of a U so that it would dip under a mercury seal. Although he gave a figure of such an apparatus, he does not seem to have ever worked with it, and his scheme needed considerable adaptation for our purpose.

Fig. 1 is an elevation and fig. 2 a general view of it. In each the lettering is the same. On an ordinary glass slide A the magnet-shaped iron object B is fixed with seccotine. At its rounded end it bears two longitudinal holes through which pass the two glass tubes C and D, while in the other direction, a short distance from the two ends, there are two vertical slits E and F facing each other across the intervening space. This piece can be most cheaply made in iron and must be lacquered all over with "Necolustre" or some such

material before it is fixed to the slide in order to prevent rusting. Then in between the limbs of the magnet there is stuck to the glass a low tin trough, G, also lacquered, which only rises to half the height of the magnet or slightly less. This will contain the mercury to form the seal. The tube D comes to an end as soon as it enters the chamber, for it will lead in the gas or gas mixture required.

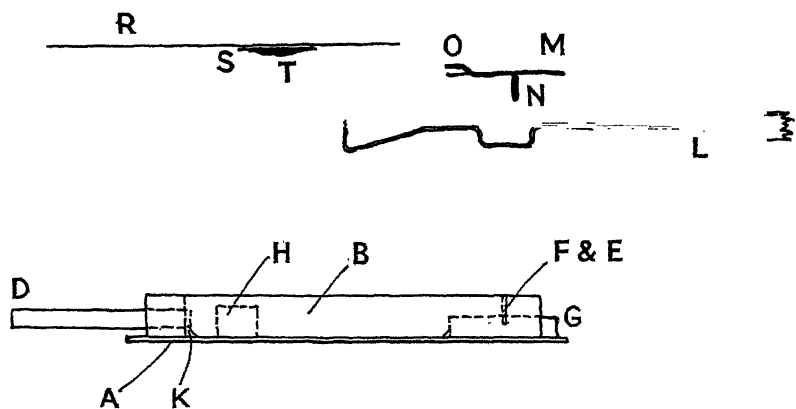


FIG. 1. ELEVATION

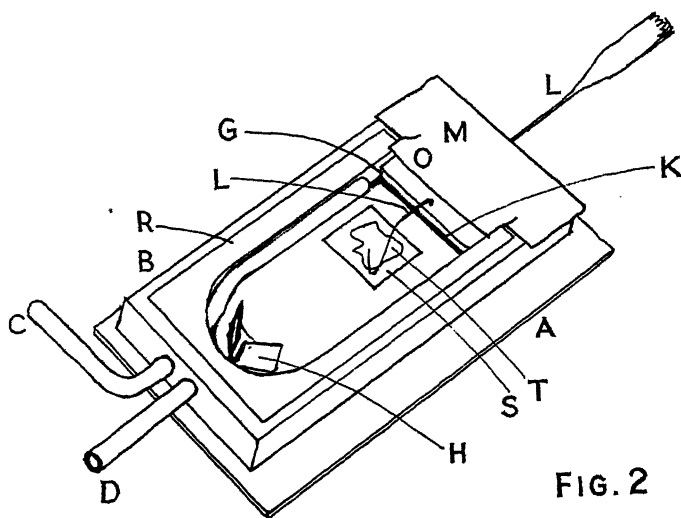


FIG. 2

The tube C, however, follows the side of the chamber right along and only ends just before reaching the mercury trough. Opposite the opening of the tube D there is a tin baffle-plate, H, lacquered, and stuck down with seccotine, so that the corners of the chamber may be well swept out by the incoming gas. The chamber is stuck rather to one side of the glass slide so that room is left for

the clip of the microscope stage to fix the whole apparatus. Round the bottom of the chamber on its inside there is a rim of paraffin wax, K, to ensure that if water is spilt or purposely placed on the floor the seccotine will not dissolve.

At L in fig. 1 is shown the micropipette which is held in the micromanipulator in the ordinary way, but bends down and up again, so as to dive under the mercury in the trough. M is a tin plate which fits down on top of the end of the chamber when the micropipette is in place. Its descending part N slides downwards in the grooves E and F, and at its inner side it is shaped as shown at O, being bent upwards so as to fit over the glass coverslip.

The glass coverslip is shown in section at R. It forms a roof for the whole chamber and is vaselined down to its sides. The crack which is left at its meeting place with M is well vaselined also. Although the construction of the pipette will permit of a considerable amount of horizontal movement of the micropipette tip, yet the freedom of movement is much more constricted than it is in an ordinary moist chamber. A "flying coverslip" is accordingly used. The hanging drop T is arranged not on the coverslip R, but on a much smaller one S, and this is attached to R by the tension of a very thin film of water between them. The position of S can therefore be adjusted before the chamber is closed according to the position of the end of the micropipette, and the remaining horizontal movements will be much reduced.

The gases used were passed through several wash-bottles containing water, and so, arriving wet at the chamber, did not dry up the hanging drop.

The amoebæ came from the same culture as those used before and were nourished by the wheat-grain method. As anaërobic cells for injection, the most suitable seemed to be some of the parasitic protozoa, and after preliminary experiments *Nyctotherus cordiformis* was adopted as the easiest one to work with. This parasite is quite common in the large intestine of the frog, *Rana temporaria*, and we found it easy to isolate from that source. A very few injections were made into *Balantidium entozoon*, a less common ciliate from the same origin, but its small size and rapid movement prevented its selection as the principal experimental animal.

The earlier of our experiments were done on *Opalina ranarum*, but it was abandoned, for it is so difficult to inject. Although it is quite as large as amoeba, yet its membrane is so tough and its thickness so small that even with the finest micropipettes it is difficult to inject it with continuous success. Worst of all, the cytoplasm seems to be so jelly-like and thick that indicators will not spread properly in it. They remain in a very localised area corre-

sponding to about 12 of the nuclei while the rest of the cell is quite uninfluenced. Recent observations of Gatenby and King (11) suggest that *Opalina* is not a ciliate at all, but a colony of flagellates, in which case it is not impossible that between the nuclei there may be membranes limiting the spread of the dye. After injection the organism continues to swim about with the aid of its cilia, and in a few moments the injected part drops suddenly out, leaving the animal shaped like a ring. This process can be repeated a number of times until the animal is quite riddled with holes.

Nyctotherus, however, was far more satisfactory. Its injection was not at all difficult provided a micropipette with a sharp end was used, and its size, though only about half that of the amoeba, was yet considerably larger than some of the eggs previously studied. One pitfall is noteworthy. If the pipette is at all blunt it does not pierce the membrane but pushes it up in front of it until the top and bottom membranes are next to each other. The cell will then retain this shape, and may appear to be injected. As the dye in the pocket thus formed will easily diffuse away, the fading may be taken for reduction. Luckily, however, there is no doubt at all when a cell is properly injected, for the dye runs all over the cell in a characteristic way. The right way of injection, then, is to have a gradually increasing pressure in the micropipette, as it rises into the cell; this will burst the lower membrane through before the pipette tip reaches the membrane at the coverslip. It is important that an injected cell continues to move about as it did before it was injected. Its cilia only stop beating when cytolysis is advanced.

As in our previous experiments, explosions occurred with all the dyes and organisms indiscriminately, so that some special type of mechanical injury would seem to be responsible.

There is no doubt that *Opalina* and *Nyctotherus* are capable of leading a strictly anaërobic existence for at least a week. The stock of organisms was prepared from the contents of a frog's large intestine by placing them in a centrifuge tube, very well stoppered and provided with a tap. The culture was exhausted by means of a Geryk pump and allowed to boil *in vacuo* for 10 minutes, after which it was filled with pure hydrogen. This process was repeated at intervals for a week, at the end of which time the animals were in excellent condition. Ringer solution was used as the fluid in which to suspend the animals, and foodstuff in moderate quantity from the large intestine was also present. Our experience has been, moreover, that excess of oxygen is toxic for these parasitic protozoa. If instead of preserving the culture in hydrogen it is left open to the air for a couple of days, it is clearly in bad

condition. The *Opalinae* are not affected so soon, but the *Nyctotherus* seem more transparent than normally, while some are spontaneously cytolysing. Moreover, they are stationary or swim about only very slowly, in contrast to those in hydrogen, which are exceedingly active at the end of the week.

We have succeeded in preparing colour photographs of many of the phenomena described in this paper. An exposure of 20 seconds was necessary, using a 1,000-candle power Edison lamp, Agfa plates, and a Leitz microscope camera.

Amœba proteus.

(1) *Injection of the series of rH Indicators.*—In the first paper of this series we found that No. 5 was partially but not entirely reduced when injected into the amœba. It was obviously a matter of considerable importance to ascertain whether all the rH indicators fell into their proper places when brought into contact with a living reducing agent. Their order is perfectly definite when they are studied away from living matter, but this regularity might not extend to the living cell and anomalous results might be obtained. If so, if the cell could reduce one indicator while failing to reduce another theoretically more easily reducible, it might well be that not all the dyes would be equally applicable to biological reactions. All the indicators might not indicate when in contact with living matter.

In our preceding paper we reported the results of micro-injecting all the dyes into the egg-cells of a sea-urchin, *Paracentrotus lividus*. There it was found that the order was well kept; Nos. 1, 2, 3, 4 and 5 were not reduced, while Nos. 6, 7, 8 and 9 all were reduced. But another complete range was desirable, and we accordingly injected all the dyes on the scale into the amœba.

The results were uniformly satisfactory. Nos. 1, 2, 3 and 4 were all unaffected by the cell, and no reduction, even after a considerable time, could be seen. No. 5, as previously described, showed a partial reduction, while Nos. 6, 7, 8, and 9 were rapidly reduced. Both Nos. 7 and 9 have a physiological pH virage, and it was interesting that our previous conclusions about the internal pH of the amœba were completely confirmed. We had assessed it at pH 7.6. No. 7 is red up to pH 8.0 or a little higher, and blue above that point; in the amœba it was distinctly red. No. 9 is red up to pH 6.75 or so, and blue above that point; in the amœba it was definitely blue.

In the account of the few preliminary experiments with methylene blue (No. 3a) which we gave in our first paper, it was said that this dye was reduced by the amœba. We have not been able to confirm this observation, although

we have made repeated attempts to do so. The earlier experiments were performed primarily to test our injection technique, and it is probable that the dye we used was too dilute to allow of proper observation of the colour changes. Clark (5) has shown that methylene blue lies on the indicator scale between Nos. 3 and 4, so that no reduction in the cell would be expected. On the other hand, there is evidence (as we have heard from Prof. Mansfield Clark in a private communication) that methylene blue—the only basic indicator in the series—may show special peculiarities in the presence of tissues.

(2) *Injection of an rH Indicator completely reduced.*—One of the principal assumptions which it is necessary to make in applying the terminology of oxidation-reduction to the living cell is that the state of affairs in it is maintained at a definite and reversible equilibrium. For this reason it was very important to determine the behaviour of the cell towards completely reduced indicators as well as towards completely oxidised ones. If in the cell the reduced glutathione is chiefly responsible for the reduction of the injected indicator, then, judging from purely *in vitro* experiments (Dixon and Quastel, 8), we might expect to get no oxidation when the completely reduced indicator is micro-injected. Again, if there is no well-poised equilibrium, the rH calculated from experiments with oxidised dyes and that calculated from experiments with reduced dyes ought to be different and variable.

In the first communication we described experiments on this point. We injected No. 5 completely reduced into *amœbæ*, and were able to see that from a perfectly colourless state a pale pink colour developed. But this change was not easy to observe, so that later on, when we had other indicators and No. 4 was available, we decided to inject this dye completely reduced. No. 4 is, of course, normally quite unreduced by this cell, so that the colour change, if it occurred, would be from colourless to deep blue. Actually, it did occur. For about five seconds the cell remained uncoloured, but then an unmistakable blue tint appeared, which deepened until the cell was as strongly coloured as if the completely oxidised dye had been introduced. This change was seen in about 30 to 40 *amœbæ*, in nearly all cases by two observers using the Leitz double eyepiece, and we were able to obtain a colour photograph of it.

A number of details are worth mention here. During each experiment a strong stream of wet nitrogen or hydrogen was flowing through the chamber. With this indicator, staining takes place neither with the oxidised nor with the reduced form of the dye, as was ascertained by injecting the drop beside the *amœba*. Even when punctured under those conditions the dye would not enter.

In the majority of the experiments the system was so free from dissolved oxygen that the hanging drop never went blue, though the amoebæ contained in it always did so. In some of these cases unwetted gas was passed through the moist chamber so that the drop should slowly dry up. Any blue colour in it would therefore have been more and more obvious, owing to the concentration of the drop, but it was never seen.

In some of the experiments the dye in the drop went blue before the amoeba did, but while it was yet faint it was outstripped by the amoeba, which took on by degrees a very deep blue colour. In this case it might be said that the water of the drop was doing as well as the protoplasm of the cell-interior, but, even so, it was quite clear that re-oxidation in the cell was possible, whether or no it was due to the external conditions. To say that the oxygen dissolved in the protoplasm is the factor responsible for the re-oxidation is no criticism of the validity of an rH calculation, for the dissolved oxygen would be only one of the factors the sum of which is the equilibrium level of oxidation-reduction potential. If it were the chief factor, responsible, then the oxidation of No. 4 by cells in an atmosphere of nitrogen is all the more remarkable.

We consider it true to say, therefore, that the cell can be thought of as having a definite, well-poised and characteristic equilibrium, which is reached by an indicator either from above, if it is more electropositive than the cell and is injected in the oxidised form, or from below, if it is more electronegative than the cell and is injected in the reduced form. If the former dye was injected reduced, it would stay reduced; if the latter was injected oxidised, it would stay oxidised. Living cells can consequently be arranged on the same scale as oxidation-reduction indicators.

(3) *Injection of pH and rH Indicators under Aërobic and Anaërobic conditions.*—To find whether the rH is influenced by the external atmosphere, and if so, to what extent, it was decided to try the effect of strict anaërobiosis on the one hand and thorough oxygenation on the other.

With this end in view the airtight chamber described in the section dealing with technique was used. The required gas was passed through the chamber during the experiment in a rather fast stream (about 1 litre every minute), and a rough calculation showed that with such a thin film of water as the hanging drop, equilibration would take place in less than a second. Table II shows the results that we obtained, and gives also the control determinations of pH .

They will be seen not to differ in any way from those at normal atmospheres. Although very difficult, it would not have been impossible to inject a completely

Table II.—*Amœba proteus*.

—	Air.			Hydrogen.			Oxygen.		
	Colour.	Result.	No. done	Colour.	Result.	No. done	Colour.	Result.	No. done
pH									
Bromthymol blue	blue	>7.5	14	blue	>7.5	8	blue	>7.5	12
Phenol red	pink	<7.7	29	pink	<7.7	12	pink	<7.7	9
rH									
No. 4.....	ox	>17	13	—	—	—	ox	>17	13
No. 5.....	partially	18	50	partially	18	7	partially	18	18
No. 6.....	red	<19	21	red	<19	10	red	<19	10

reduced indicator under these strict conditions of gas concentration, but we did not do so, for there seems no reason to suppose that the external atmosphere should influence the oxidising power of the cell if it does not influence the reducing power.

The rH of the cell-interior seems to be independent of the external oxygen concentration. In our first paper we drew attention to various investigations in the literature which went to show that in many cases where the circulation does not enter as a limiting factor, the oxygen consumption of the individual organism was widely independent of the oxygen concentration of the environment. Since then further work has been done on these lines, and we have learnt of other older papers bearing on them. Thunberg (26) found that the larvæ of *Tenebrio molitor*, the meal-worm, were able to preserve their gas exchange unaltered within the limits of 100 to 30 per cent. oxygen. Lund (17) found great independence on the part of planarian worms, and in a later paper (18) on the part of *Paramœcium* also. Godleffski (12) obtained results showing great independence in frog's egg, but Parnas and Krasinska (23) have since published results which do not confirm this.

Many researches have brought out data varying with the animal used. Henze (14) repeated the work of Warburg (30) on sea-urchin eggs, and came to the conclusion that they consumed more oxygen the more there was in their environment. He considered that Warburg's eggs had been insufficiently shaken. A similar dependence was found for *Sipunculus nudus*, a gephyrean worm, and for various Anthozoa. Asphyxia would not explain it, for on raising the oxygen concentration the oxygen utilisation also rose. Henze made the observation that in one of the varying animals, *Sipunculus*, the CO₂

production was not raised in high environmental oxygen concentrations, though the oxygen intake was. Later, Dakin and Dakin (7) found plaice eggs to be very dependent. McClendon (19) found similar variability with *Cassiopeia*, and Amberson, Mayerson, and Scott (1) with *Homarus*, *Limulus*, *Nereis*, and *Callinectes*.

The *in vitro* work has, however, all been on the side of independence. Hamburger and Szent-Gyorgy (13) found that washed muscle took up just the same amounts of oxygen for oxidising paraphenylenediamine in one atmosphere of oxygen as in one-twentieth of an atmosphere of oxygen. Wels (31) found that the oxygen uptake of adult guinea-pig diaphragm muscle tissue was identical from 100 to 40 per cent. oxygen.

Thus, in some cases, it seems to be true that living cells take in the same amount of oxygen whatever their external concentration may be, while in other cases that amount seems to depend directly on the outside conditions. In the former the semi-permeability of the membrane, or some similar factor limiting the oxygen uptake, will be a powerful cause tending to keep the rH constant; and in the latter, if the rH does maintain its constancy, this may result from a true poisoning action, due to the S-shaped form of the oxidation-reduction titration curve, or to a general increase of metabolic rate, the same substances being oxidised, or to some other circumstance.

Nyctotherus cordiformis.

(1) *Determination of the pH and rH under Aerobic Conditions.*—These results are summarised in Table III. Bromthymol blue on injection gave a pure green colour which, matched against buffer and dye in micro-test-tubes, corresponded to pH 7.1 to 7.2. After 20 to 160 seconds the green gradually changed to yellow as cytolysis proceeded. Bromcresol-purple gave a purple colour which persisted for a long time.

As regards the oxidation-reduction indicators, no reduction of Nos. 4 and 5 could be seen, but No. 6 was clearly decolorised. The cells used for these injections had been exposed to the air for not more than 24 hours; they were active and appeared to be in good condition.

(2) *Determination of the pH and rH under Anaerobic Conditions.*—The technique for obtaining anaerobic conditions was exactly the same as that used for the amoebae. The results are set out in Table III. It will be seen that the pH is unaltered, but a striking change has taken place in the reduction intensity. No. 1 now appears to be the only indicator of the series unreducible by the organism. It has become more reducing by practically 10 rH units.

Table III.—*Nyctotherus cordiformis*.

	Aërobic Conditions.			Anaërobic Conditions.		
	Colour.	Result.	No. done.	Colour.	Result.	No. done.
<i>pH</i>						
Bromthymol blue	green	7·1	25	green	7·1	15
Bromcresol purple	purple	>6·6	12	purple	>6·6	14
<i>rH</i>						
No. 1	—	—	—	ox	> 9	17
No. 2	—	—	—	partially reduced	<10	26
No. 3	—	—	—	red	<11	20
No. 4	ox.	>18	18	red	<17	33
No. 5	ox.	>19	12	red	<18	31
No. 6	red	<20	7	—	—	—

The *rH* dyes had remarkably little toxic effect on *Nyctotherus*, whether aërobic or anaërobic. An hour after injection the cells would in some cases be observed swimming about as actively as before.

With the indigotines it was observed that the nucleus remained colourless for the first few seconds after the injection of the cell (if the nuclear membrane was not pierced) and then gradually took up the dye, so that after a short interval a pale blue nucleus in a colourless cell might be seen. With No. 3 the nucleus soon faded, but with No. 2 hardly at all before cytolysis. This passage of dye into the nucleus was, however, certainly not sufficient to account for the decolorisation seen in the cytoplasm.

It was found that if the cell was caused to cytolysed by pricking, this would be followed by a successive cytolysis of the cells in the immediate neighbourhood. This phenomenon may be compared to that observed recently by Drzewina and Bohn (9). In thickly populated cultures of *Convoluta roscoffensis*, *Anthea cereus*, and *Paracentrotus lividus* eggs, cytolysis appeared to be infectious. They showed that this was due to a local production of acid by the cytolysing cell and a resulting cytolysis of its neighbours. In thin cultures the other individuals would tend not to be affected.

Opalina ranarum.

Nos. 1, 2, 3, 4, and 5 were injected into these cells under anaërobic conditions. No. 1 seemed to be particularly toxic for this organism, and the injected part was cut off so rapidly that little information could be gained. In the other four cases, however, fading was quite definite and often so rapid that reduction,

not diffusion, seemed to be the only explanation. But as enough dye to colour only about one-tenth of the cell could be introduced at any one injection, it was impossible to deny that fading might be due to diffusion of this small quantity throughout the cell. The dye might then become so dilute as to be invisible. No. 5 was injected under aërobic conditions, but here the results were very uncertain. Fading took place sometimes, but always slowly, and there were other difficulties of interpretation.

Discussion.

One of the most interesting points studied in this work was the effect of the external atmosphere, on the amoeba on the one hand and on *Nyctotherus* on the other hand. The amoeba, of course, disintegrates if kept in an atmosphere deprived of oxygen (24, 29) for any length of time; but in an atmosphere of hydrogen for 30 minutes its activity is unaffected, and this treatment has no effect on its reduction intensity. With *Nyctotherus*—and possibly with *Opalina*—a definite difference in reducing power is found between the aërobic and anaërobic conditions, but we have not yet ascertained how long an exposure to either of these environments is necessary to establish a definite rH . It seems that in the facultative anaërobe the oxidation-reduction potential can be adjusted to the environment, while in the strict aërobe changes in the environment which would lead to such an alteration in the rH bring about death.

There is another important point which needs discussion. In the case of any particular indicator there may be doubt as to whether it does or does not function in the absence of a catalyst. It is not possible to micro-inject colloidal platinum, so that we have to be content with less direct evidence. As has been described, several cases of partial reduction have come to light—the amoeba with No. 5, the egg-cells of *Ascidia mentula* with No. 6, *Nyctotherus* anaërobically with No. 2—and in these cases it is clear that the indicator is acting. We should then expect the indicators below in the series to be unaffected, whether functioning or not, while the indicators above, if functioning, should be reduced. This is just what is found. With *Nyctotherus*, moreover, there is evidence that the whole series, with the exception of No. 1, is able to function in the cell. It seems therefore reasonable to assume for the present that lack of reduction in the cells studied is not due to an absence of the necessary catalyst.

The results described are, of course, based on the average colour of the whole cytoplasm of the cell, observed under a quarter or a sixth lens. It is very likely that small localised areas, if studied closely, would be found to show special behaviour; indeed, in *Nyctotherus* the nucleus was seen to show

certain constant differences from the cytoplasm, as also that of the eggs of *Ophiura lacertosa*. In other cases we have often noticed granules and vacuoles which showed a different colour or a different depth of colour from the rest of the cell. No. 7 in the amoeba is an instance of this, for although the cell as a whole was red, some of the vacuoles were always blue, indicating a pH higher than 8.0. But great care will be needed to determine whether these differences show any constancy, and as the chemical composition of these areas is probably highly specialised, it is likely that adsorption, staining, and other effects come in, making it impossible to compare their behaviour towards the indicators directly with the behaviour of the cytoplasm.

Finally, mention may be made of recent work in which the oxidation-reduction potential was measured of a system not inside the cell, but in a medium where the living cell (or an enzyme obtained from it) was present as activating agent. Thunberg (27) used an equimolecular mixture of succinic and fumaric acids in the presence of succinodehydrogenase, under anaërobic conditions; he found that in these circumstances methylene blue was partially decolorised, a definite equilibrium being reached. From the degree of reduction of the methylene blue, the E_h of the system was calculated. According to this E_h , indigotinemonosulphonic acid should not be reduced, and, in fact, it was found not to be.

Summary.

1. Oxidation-reduction potential indicators exhibit no anomalies when injected into *Amoeba proteus*. It appears, therefore, that all the dyes at present in use on the rH scale may be employed with biological material.
2. The amoeba is capable of oxidising the leuco-form of indicators of lower oxidation-reduction potential than its own.
3. The rH of the amoeba is probably widely independent of the concentration of oxygen in the external atmosphere.
4. *Nyctotherus cordiformis* (an anaërobic protozoon) has an internal pH of 7.1, and an internal rH of 19.0 to 20.0 under aërobic conditions, while under anaërobic conditions the latter value changes to rH 9.5-10.5.

The writers' thanks are due to Prof. Sir F. G. Hopkins for his constant interest and encouragement during this research.

REFERENCES.

- (1) Amberson, Mayerson, and Scott, 'Journ. Gen. Physiol.,' vol. 7, p. 171 (1924).
 - (2) Barber, 'Philippine Journ. Sci.,' vol. 9B, p. 309 (1918).
 - (4) Chambers, 'Anat. Record,' vol. 24, p. 1 (1922).
 - (5) Clark, 'U.S. Public Health Reports,' Reprints Nos. 823, 826, 834, 848, 904, 915, 1017.
 - (6) Clark, 'Journ. Wash. Acad. Sci.,' vol. 14, p. 123 (1924).
 - (7) Dakin and Dakin, 'Brit. Journ. Exp. Biol.,' vol. 2, p. 315 (1925).
 - (8) Dixon and Quastel, 'Trans. Chem. Soc.,' vol. 123, p. 2943 (1923).
 - (9) Drzewina and Bohn, 'Comptes Rend. Acad. Sci.,' vol. 181, p. 692 (1925).
 - (11) Gatenby and King, 'Nature,' vol. 116, p. 712 (1925).
 - (12) Godleffski, 'Arch. f. Entwicklmech.,' vol. 11, p. 603 (1901).
 - (13) Hamburger and Szent-Gyorgy, 'Bioch. Zeitschr.,' vol. 157, p. 298 (1925).
 - (14) Henze, 'Bioch. Zeitschr.,' vol. 26, p. 255 (1910).
 - (15) Koenig and Brodhun, 'Sitz. d. Ak. wiss. Berlin,' p. 641 (1889).
 - (16) Laurens and Hamilton, 'Amer. Journ. Physiol.,' vol. 65, p. 547 (1923).
 - (17) Lund, 'Biol. Bull.,' vol. 41, p. 203 (1921).
 - (18) Lund, 'Amer. Journ. Physiol.,' vol. 45, p. 351 (1917).
 - (19) McClendon, 'Journ. Biol. Chem.,' vol. 32, p. 275 (1917).
 - (20) Needham and Needham, 'Roy. Soc. Proc.,' B, vol. 98, p. 259 (1925).
 - (21) Needham and Needham, 'Roy. Soc. Proc.,' B (1926) (*in the press*).
 - (22) Needham and Needham, 'Comptes Rend. Soc. Biol.,' vol. 113, p. 503 (1925).
 - (23) Parnas and Krasinska, 'Bioch. Zeitschr.,' vol. 116, p. 108 (1921).
 - (24) Pütter, 'Zeitschr. f. allg. Physiol.,' vol. 5, p. 566 (1905).
 - (26) Thunberg, 'Skand. Arch. f. Physiol.,' vol. 17, p. 133 (1905).
 - (27) Thunberg, "Proc. Ist Nordic Congress of Physiology," 'Skand. Arch. f. Physiol.,' vol. 46, p. 339 (1925).
 - (28) Utthof, 'Arch. f. Ophthalmol.,' vol. 34, p. 14 (1888).
 - (29) Verworn, 'Denkschr. Jena. Ges.,' vol. 11, p. 561 (1904).
 - (30) Warburg, 'Zeitschr. f. Physiol. Chem.,' vol. 57, p. 4 (1908).
 - (31) Wels, 'Pflüger's Archiv,' vol. 209, p. 32 (1925).
-

The Mechanism of Ciliary Movement.—V. The Effect of Ions on the Duration of Beat.

By J. GRAY, King's College, Cambridge.

(Communicated by Prof. Stanley Gardiner, F.R.S.—Received January 19, 1926.)

In a previous paper (Gray, 1924) it was shown that the whole series of events which accompany continuous ciliary movement is divisible into at least three phases: (i) A reaction which is sensitive to monovalent cations (particularly the hydrogen-ion); any interference with this mechanism involves a change in the rate of beat of the cilia, and ultimately leads to a corresponding change in the oxygen consumption of the cells. (ii) A mechanism which is brought into operation by the presence of an activating acid substance. (iii) A reaction of an oxidative nature which removes some factor which is the direct result of activity and which, if allowed to accumulate, will inhibit the activity of the whole ciliary apparatus. The present paper deals primarily with the first of these processes. For this purpose the lateral epithelium on the gills of *Mytilus edulis* form a convenient material, since under appropriate conditions the cilia of these cells can (for many hours after excision of the gill) either be maintained in an active state of movement or be kept quite motionless.

If the gills of a healthy animal be excised and examined at once under the microscope, the lateral cilia are seen to be in active movement, and exhibit a very beautiful metachronial rhythm which passes up and down the two sides of the gill-filaments. If these gills are now thoroughly washed with sea-water the lateral cilia sooner or later come to rest; the period which elapses before quiescence varies considerably with different individuals. It is usually about 15 mins., but it may be considerably longer in the case of well-fed mussels. It is to be concluded that the amount of available energy in the lateral epithelium is usually sufficient to maintain activity in sea-water for a strictly limited period. This is in marked contrast to the frontal epithelium, which remains active in sea-water for very long periods; in this case it may be assumed that the supply of energy is still being maintained from the glycoprotein (Gray 1924).

When the lateral epithelium has exhausted its supply of available energy in sea-water, it can be re-activated in three ways (1) by increasing the concentration of potassium-ions present; (2) by the presence of veratrin; (3) by increasing the concentration of hydroxyl-ions (Gray, 1924). To these must now be added (4) by reducing the concentration of magnesium-ions present.

Stress has been laid on the fact that although magnesium plays an important rôle in maintaining the stability of the tissue as a whole, yet the frontal cilia of *Mytilus* will beat apparently unchanged for very long periods, either in the absence of magnesium or in the presence of a great excess (Gray, 1922). A fundamental rôle of magnesium can, however, be demonstrated in the case of the lateral cilia. If gills are placed after excision into magnesium-free sea-water these cilia do not stop, but show a marked increase in their rate of beat. The cessation of movement which occurs in normal sea-water is primarily due to the presence of magnesium. The increased rate of beat which occurs in the absence of this metal is rapidly attended by reduction in the amplitude of the stroke, a loss of vigour, and often by irregularities in the transmission of the waves over the epithelium. On replacing the magnesium two significant changes occur (1) within five or ten minutes the rate slows down, the amplitude increases, and the rhythm becomes steady, (2) the cilia remain active in sea-water for many hours.* Exactly the same phenomena can be produced from tissue whose lateral epithelium has come to rest in sea-water. The transference of such tissue to any solutions lacking magnesium initiates activity of the lateral epithelium. By adding magnesium when rapid activity has set in, the speed is reduced, and the cilia remain active for prolonged periods. It is obvious that a comparatively short exposure to solutions lacking magnesium has produced two changes: (1) it liberates in the cell a supply of available energy which lasts for a prolonged period, even when magnesium is afterwards present, (2) it quickens the rate at which this energy is expended by the cilium, but this only occurs as long as magnesium is absent.

It has already been shown that in the presence of magnesium the lateral cilia can be activated by adding potassium (Gray, 1922), and there can be no doubt that these two metals operate on the same point in the mechanism. If tissue exhausted in sea-water is re-activated by adding potassium, exactly the same phenomena occur as on removal of magnesium. The lateral cilia begin to move with an increasing speed, the amplitude falls, and the rhythm may become irregular. On replacing in normal sea-water the rate falls rapidly and a regular rhythm is sustained for as much as 18 hours.

The most characteristic feature of potassium is its power of antagonising magnesium inhibition. Potassium in itself is not essential for movement, because if magnesium is absent the lateral cilia will beat in the absence of potassium.

The presence of 0.04 molecular potassium will maintain the activity of the

* They will also beat for several hours in $M/2 \text{ MgCl}_2$.

cilia even in the presence of quite high concentrations of magnesium. The following table shows the degree of antagonism between the two metals :—

Solution: NaCl + 0.5 Mol. CaCl ₂ + 0.005 Mol.	Conc. of K. required for prolonged activity of lateral cilia.
0 MgCl ₂	0
0.01 Mol.	0.02 Mol.
0.02 "	0.03 "
0.04 "	0.03 "
0.06 "	0.04 "
0.08 "	0.04 "
0.10 "	0.04 "

It may be noted that the power of magnesium to resist the action of potassium reaches a maximum about the concentration found in sea-water, viz., 0.05 mol., whereas the concentration of potassium which will resist the full inhibiting powers of magnesium is about that found in the blood.

Why potassium should differ so fundamentally from sodium in its antagonism to magnesium is unknown. It is, however, parallel to the fact that in the presence of excess of potassium, magnesium is unable to stabilise the intercellular matrix and prevent the uptake of water by the cells; whereas if potassium is replaced by sodium, magnesium has a well-marked stabilising action (Gray, 1922A). It looks as though potassium can eject magnesium from some position, whereas sodium cannot do so.

Neither the action of magnesium nor of potassium is due entirely to the specific nature of its ions. Magnesium can only affect the supply of available energy when acting on a background of sodium. Thus, lateral activity is maintained for prolonged periods in solutions of non-electrolytes containing large quantities of magnesium, and cells exhausted in sea-water become active in such solutions.

Solution	Conc. of Mg ..					
	0	0.025	0.05	0.075	0.10	
M. Glycerine						++++=very rapid beat.
pH 7.8	+++	++	++	++	++	++=normal beat.
M/2 NaCl	..	++	0	0	0	0=inactive.

This indicates that both sodium and magnesium inhibit the replenishment of available energy, although magnesium is by far the more powerful of the two.

The restraining influence of magnesium on the speed of the beat is, however, exerted even in solutions of non-electrolytes, although its action is definitely

limited to maintaining a slow steady rhythm. The only way in which lateral or other cilia can *rapidly* be brought to rest is by adding hydrogen-ions.

The effect of veratrin on lateral cells exhausted in the presence of magnesium is essentially the same as that of potassium, although the duration of activity on removing the veratrin is not usually so long (it is about 30 minutes). If the tissue is again exposed to veratrin and replaced in sea water another period of activity takes place.

In respect of both the frontal and lateral cilia there is a marked difference in the properties of magnesium and calcium. The latter metal appears to be the only metallic cation which is specifically necessary for the prolonged activity of the frontal cilia. In Ca^{++} free sea-water, pH 8.0, these cilia beat for several hours, but in slightly more acid water movement ceases fairly rapidly (Gray, 1924). Cessation of movement is preceded by a slowing of the rate until the cilia eventually become motionless. On adding calcium the normal rate of activity is gradually recovered, although in the presence of alkalis recovery is very rapid. In the case of the lateral cilia, calcium also appears to be essential, although they are less sensitive to its absence. In all the solutions mentioned in this paper calcium has been present in the concentrations of normal sea water, unless special reference is made to the contrary. The type of cessation of movement due to lack of calcium is very similar to that produced by excess of magnesium, and the fact that a lack of calcium can be compensated for some time by the presence of potassium, or by alkali, suggests that calcium is required for the formation of an energy reserve from the glycoprotein. Against this view is the fact that stoppage of the frontal cilia in lack of calcium involves no reduction in the rate of oxygen consumption (Gray, 1924), whereas stoppage of the lateral cilia by the action of magnesium involves a reduction in the oxygen consumption. The most striking point is, however, that the inhibiting powers of magnesium are not shared by calcium. The presence of the latter metal prolongs activity and does not influence the rate of beat. In view of the fact that the two metals act differently on the oxidative mechanism, further work is required before any closer analysis can be attempted.

In the absence of both magnesium and calcium the ciliated cells all absorb water by imbibition. This phenomenon is described elsewhere (Gray, 1925), and is largely due to the fact that the divalent cations exert a strong antagonistic effect against the swelling action of hydroxyl-ions. Complete immunity from swelling at pH 8.0 is only effected when sodium, potassium, magnesium and calcium chlorides are present, as in sea-water. In all other

solutions inhibition can be observed, although it appears very much more slowly when magnesium and calcium are present. If tissue, in which the lateral cilia have been exhausted in sea water, is transferred to any solution in which inhibition occurs, there is always a period before swelling is very obvious during which the lateral cilia become active. The process of inhibition appears to favour the liberation of a supply of energy in the cells. Since the length of the latent period before activity begins appears to be correlated with the period which elapses before inhibition is detectable, it appears to be an invariable rule that lateral activity precedes the inhibitional swelling of the tissue.

Discussion.

The energy which is expended by the cilia of *Mytilus* appears to be derived from some form of glycoprotein (Gray, 1924), but in order that such a substance should yield a supply of available energy it must first be converted into some simpler derivative, and in order that continuous ciliary activity may be maintained this degradation of the glycoprotein reserve must also go on continuously. The fact that in sea water the lateral cilia normally come to rest after about 15 minutes receives its simplest explanation on the assumption that the store of available energy in the cells is only sufficient for a limited period of activity, although a plentiful supply of glycoprotein is still present. In sea-water the formation of such a store of available energy is prohibited, but by exposing the tissue for a short time to an absence of magnesium, or to 0.04 mol. potassium, to hydroxyl ions, or to veratrin, a rapid formation of available energy occurs, so that on transference of the tissue to normal sea water prolonged activity is maintained.

The cessation in the formation of available energy which is brought about by magnesium is parallel to its effect in striated muscle (Embsden, 1923), and in the latter case it can be shown that it operates on the conversion of glycogen into lactacidogen. Equally parallel are the antagonistic effect of potassium, the "stimulating" action of veratrin, and the effect of those agents which produce inhibitional swelling in the cell.

Perhaps the most significant feature displayed by these experiments is this very close parallel, which exists between cilia and other forms of contractile mechanisms. The effects of magnesium and potassium on the lateral cilia of *Mytilus* are exactly the same as on the rhythmical contraction of the isolated disc of *Cassiopea* (Mayer, 1906); while the depressant action of magnesium is in keeping with Mines' (1912) observations on the heart of *Pecten*. The depressant effect of magnesium observed by Mines was caused by solutions

containing no potassium. It would be of interest to know whether the heart of *Pecten* is less sensitive to magnesium if potassium is present.

At present the only outstanding difference between cilia and rhythmical muscular elements is the fact that in some cases potassium appears to depress the latter instead of acting as a stimulant.

Whilst the duration of a period of activity depends in the long run on the amount of available energy, the speed of the beat depends on the rate at which this energy is transferred to the contractile mechanism itself. In this case hydrogen-ions and magnesium-ions are the dominating influences. At the pH of sea-water magnesium exhibits a characteristic power of restraining the rate at which the cilia beat. It slows the beat, and thereby maintains it at full power and amplitude.

The various ways in which the lateral cilia of *Mytilus* can be made to undergo a prolonged period of activity suggest that the factor involved is a change in the state of some colloidal system on the alkaline side of its isoelectric point. The more highly ionised is the glycoprotein the more rapidly will it undergo changes in the presence of a suitable enzyme, and give rise to supplies of available energy. At present, however, there is no evidence that such systems display the peculiar characteristics of potassium, so that further speculation is hardly profitable.

It is of considerable significance to note that the experiments described in this paper show that the reaction of the cells to such cases as magnesium differs, according to whether the ion approaches the cell from the proximal surface or not. The distal surface is normally in contact with sea-water, and the proximal surface is normally bathed in blood—it is only when the proximal surface is also bathed with magnesium that the ciliary mechanism is affected in the way described. In other words, in the case of the intact animal the presence of magnesium in the external medium stabilises the intercellular matrix (see Gray, 1925) but does not affect the movement of the cilia, but the same amount of magnesium in the blood would be sufficient to cause arrest of the lateral cilia.

Summary.

1. The ciliated cells on the lateral epithelium of *Mytilus* gills contain a supply of available energy sufficient to maintain activity in sea-water for a limited period. This supply can be augmented in four different ways:—(a) By treatment with veratrin, (b) increasing the concentration of hydroxyl-ions,

(c) increasing the concentration of potassium, (d) decreasing the concentration of magnesium.

2. In the presence of sea water containing the normal concentration of sodium, potassium and calcium the presence of magnesium prevents the augmentation of the available supply of energy. Potassium exerts a strong antagonistic action to magnesium.

3. Magnesium controls the rate at which the lateral cilia beat, as well as the duration of their activity. The presence of magnesium is characterised by a powerful and steady beat with very regular metachronial rhythm.

REFERENCES.

- Embden, G., 'Naturwiss.', vol. 2, p. 985 (1923).
Gray, J., 'Roy. Soc. Proc.', B, vol. 93, p. 104 (1922).
Gray, J., 'Roy. Soc. Proc.', B, vol. 93, p. 122 (1922A).
Gray, J., 'Roy. Soc. Proc.', B, vol. 96, p. 95 (1924).
Gray, J., 'Brit. Journ. Exp. Biol.', vol. 3 (*in press*) (1925).
Mayer, A. G., 'Carnegie Instit., Wash.', Publ. No. 47 (1906).
Mines, G. R., 'Journ. of Physiol.', vol. 43, p. 467 (1912).
-

*The Influence of Muscle Work on Metabolism in Varying
Conditions of Diet.*

By E. P. CATHCART, F.R.S., and W. A. BURNETT (late Lawrence Student),
Institute of Physiology, Glasgow University.

(Received January 16, 1926.)

One of the most widespread of common beliefs is that when hard muscular work is to be done an abundant supply of protein in the diet is desirable, if not, indeed, necessary. This belief has been most energetically combated from various points of view. It has been held by many that, although muscle is admittedly largely composed of protein and other nitrogen-containing materials, in the course of muscle work these substances do not participate in any way and hence there is no necessity to make good a store that is not depleted. Others, like Chittenden (1) and Hindhede (2), attack the belief on what might almost be called social grounds. Both of these workers dissent from even the commonly accepted standards of protein intake, advocating an intake on a much lower level, on the ground that low protein intake conduces to health and happiness. Despite the chill of scientific argument on the one hand, and the warmth of a fervent belief on the other, the man in the street consistently refuses to accept the dicta of either party. And the interesting fact emerges from the study of investigations like those of McCay (3) that there does seem to be some connection between race virility and racial dietetic habits. Campbell (4) in his work at Singapore confirmed the more comprehensive work of McCay, viz., that the more virile peoples are large consumers of protein.

Whether or no it was based on the actual needs of the soldiers, the dietaries of the various allied armies, as regards their protein content, followed more or less closely the results of the experimental investigations of the food requirements of soldiers engaged in marching carried out by Melville and his co-workers (5). These investigators used two diets, one containing per day 190 grams of protein, of which 100 grams was in the form of corned beef and 75 grams in the biscuit consumed, and the other contained 145 grams, supplied mainly in the form of fresh meat and bread. Melville stated that in his opinion the 190 grams allowance was ample, if not over generous, but that the 145 gram diet was "as low as it is advisable to go and might well be increased, especially when hard work is demanded of men under conditions of exposure."

Rubner (6), in one of his contributions to the general problem of dietetics, when discussing the Voit standard of 118 grams of protein, admitted that although he thought this allowance gave an excess of protein in the average diet, yet it might be considered a factor of safety, and maintained that this standard should be adopted when fixed general diets had to be constructed.

Of course it must not be forgotten in the consideration of such work as this, that protein may be of importance in the dietary, not merely on account of its highly specialized content of nitrogen, but also on account of its very high specific dynamic action. Now, whatever other function this special metabolic stimulating capacity of protein has, we have no right to assume that the extra liberation of heat—the end result—is also the expression of the actual series of changes which have resulted from the ingestion of protein. We can ask, but we cannot answer, the question as to the antecedent phenomena which eventually lead to an excess liberation of energy in the form of non-utilizable heat. Here, as elsewhere, heat is simply the final degradation product in the chain of metabolic events. It is possible, indeed it is highly probable, that the specific dynamic action exercised in so marked a degree by protein, quite apart from the useful gain of actual heat for those exposed to peculiarly rapid loss of heat, plays a real part in stimulating tissue activities in general. There is the further point that the work of Zuntz, Rubner (7) and others appears to furnish good evidence that protein on ingestion, for instance in muscle work and in growth, does not invariably exhibit its specific dynamic property.

Lusk (8), who of all modern workers has gone furthest into this question, has come to the conclusion that “the specific dynamic action of protein consists in a specific chemical stimulus of the cellular protoplasm, which is independent of the oxidation of the material through which the stimulus is applied.” We are in agreement with the conclusion of Benedict and Carpenter (9) when they stated that “we are firmly convinced that the excess heat produced from the ingestion of protein or carbohydrates, like sugars, may not properly be considered as a purely waste process, but that it is far more logical to consider it as a general stimulation of all cells in preparation for the drafts of muscular activity.”

It is very difficult to obtain any satisfactory evidence which will enable us to reach anything like a definite conclusion on this abstruse, but at the same time interesting and important, point. There is in our opinion no doubt about one fact, viz., that the stimulation of metabolism which results from the ingestion of protein does not pass off immediately. The ingestion of protein, whether by its specific dynamic action or no, would seem to stimulate

definitely intracellular metabolism. The following table (10) shows this clearly :—

Basal Metabolism.				O ₂ intake in c.c. per min.	Cals. per sq. metre.
Mean of all experiments on subject M.				242	39·8
Ordinary mixed diet	252	40·9
Carbohydrate diet	210	35·6
Fat diet	212	35·1
Protein diet	274	44·8

It will be noted that the basal metabolism as determined when the subject was on an ordinary mixed diet agrees very well with the average value determined by Du Bois, viz., 39·7 cal. per square metre surface area per hour, whereas the value obtained when the subject had been kept on a protein-rich diet is 44·8—a figure well above the average. On the other hand, the basal values determined when the subject lived on a carbohydrate-rich or a fat-rich diet are well below the average value. Yet Rubner, Lusk and others hold that both carbohydrates and fat have a definite, although perhaps small, specific dynamic action. The explanation of the variance may lie in the fact that the specific dynamic action exercised by these substances is more evanescent in character, or that the enhancement of metabolism, which is found to follow the ingestion of fat and carbohydrate, is not a true specific dynamic action comparable with that induced by the ingestion of protein. Lusk (*loc. cit.*) has recognized this differentiation, and prefers to call the result in the case of fat and carbohydrate the metabolism of fat and carbohydrate plethora. He has also shown in another way that the specific dynamic action of protein differs from that of carbohydrate. He noted that if the energy utilized in the performance of a definite amount of work were determined before and after the ingestion of meat protein, it was found that in the latter case “the energy requirement for work in the first instance is superimposed upon the metabolism as induced by the specific dynamic action of the protein in the second instance. This strictly differentiates between the character of the specific dynamic action of protein and glucose.” After the ingestion of glucose in the place of protein in work experiments Lusk found that there was no enhancement of cellular metabolism upon which the metabolism requisite for muscular effort had to be superimposed.

This evidence apparently indicates that protein plays another important rôle, quite apart from the necessary supply of nitrogen and sulphur in the

various intracellular, metabolic phenomena. Benedict and his co-workers (11) too, in their study of under-nutrition, state that "one of our unsolved problems in the research is the relationship between body protein and metabolic level. If the lowered general metabolism is due to the absence of protein in the body—and evidence points strongly towards this—we may then argue that, while in times of stress the minimum and lower level is justifiable and reasonably safe, in times of plenty the optimum is a higher protein level."

The problem also requires examination from the catabolic aspect, and the influence of muscular work on the output of nitrogen in the urine has on this account been repeatedly investigated. The consideration of the literature on this subject led Lusk (than whom few are better qualified to judge) to conclude that muscle work was without influence either on the qualitative or quantitative aspects of protein catabolism. It is difficult to understand how Lusk reached this very definite conclusion. One of us (E. P. C.), who in connection with the experiments about to be described re-read practically all the available literature on the subject from 1865 onwards, has come in a recent paper (12) to the very definite conclusion that muscular activity does induce quite a definite, but usually a small, rise in the output of nitrogen. There is no evidence whatsoever that the protein breakdown, as was formerly believed, forms the main, or even an important, source of energy for muscular contraction.

Present Work.

The whole series of experiments about to be described were carried out on a single subject, one of the present authors (W. A. B.). The subject, aged 27 years, weighed approximately 79 kilos., was 182 cm. high, and had a surface area of about 1.99 sq. metres. At the start of the experimental period he was in excellent physical condition, and he continued fit throughout. The work was all carried out on the hand-lever ergometer (13), on which the subject had much previous experimental experience. The scheme of work was uniform in all the experiments, with the exception of those on the pure fat diet. The subject consumed one of the fixed diets until nitrogen equilibrium was reached; still taking the standard diet, he then performed a given amount of work, approximately 25,000 kilogrammetres in an hour, for six successive days; thereafter he continued on the same diet until the base-line nitrogen output was again reached, *i.e.*, a pre-work, work, and a post-work experiment was carried through on the same fixed diet. The rest of the day the ordinary routine laboratory work was done. The work performed on the ergometer could not be considered excessive for the subject, although it would be regarded

as very hard by the ordinary man, as in the hour's period the work done was equivalent to a fourth of what is generally held to be the average day's output for a labourer.

The diets tested, although all of approximately equal energy value (calorie value), varied markedly in composition. Four diets in all were utilized in the eleven experiments of the main series here dealt with. The daily diets were as follows :—

- (i) Wholemeal wheat flour (made up in the form of scones, home-baked) 16 oz., margarine 4 oz., sugar 3 oz., marmalade 2 oz.
- (ii) Wholemeal wheat flour 12·5 oz., cheese 3·5 oz., margarine 3 oz., sugar 3 oz., marmalade 2 oz.
- (iii) As in (ii) with the addition of 8 gr. of meat extractives.
- (iv) Wholemeal wheat flour 12 oz., lean beef 8 oz., margarine 3 oz., sugar 3 oz., marmalade 2 oz.

The approximate caloric value of all these diets is 2,900 calories. Each day, in addition to the foodstuffs detailed above, the subject ate either an apple, a lemon, or an orange.

That the diets were ample is clearly shown by the steadiness of the body weight throughout the whole series of experiments. At the start of the experiments the choice of the *amount* of food was left to the subject. The subject gained steadily from 78·5 kilos. at the start to 80·1 kilos. at the end—a period of twelve months. There was practically no loss of weight during the 6-day work periods, as the following Table I clearly shows :—

Table I.—Body Weight in Kilos. during Work Periods.

Exps.	I.	II.	III.	IV.	V.	VI.	VII.	VIII.	XI.	XII.	XIII.
Start	78·5	79·0	79·9	80·5	80·7	81·3	79·8	79·9	79·2	80·6	80·6
End	78·8	79·8	79·8	79·9	80·1	81·2	79·6	80·4	79·9	80·0	80·1

PART I.

Influence of Muscular Work on the Distribution of Nitrogen in the Urine.

As Tables II and III show there is in each experiment a slight rise in the output of nitrogen during the work period, and, as has been generally found before, this rise is continued into the post-work period. The figures given

Table II.

State.	Nitrogen gr.				Chloride gr.	Total Sulphate gr.	Total P_2O_5 gr.	Diet.
	Total.	Ammonia.	Uric Acid.	Creatinine.				
Pr. W.*	8.55	0.374	0.207	0.547	—	—	—	I.
W.	10.10	0.499	0.184	0.559	—	—	—	
Po. W.	9.15	0.529	0.154	0.548	—	—	—	
Pr. W.	8.65	0.482	0.162	0.545	2.90	2.024	1.936	I.
W.	10.04	0.515	0.210	0.575	3.59	2.129	2.173	
Po. W.	9.05	0.447	0.158	0.555	4.83	2.082	2.039	
Pr. W.	16.58	0.755	0.229	0.601	6.36	2.728	2.966	II.
W.	19.52	0.738	0.240	0.633	6.94	2.927	2.808	
Po. W.	16.31	0.630	0.206	0.622	6.53	2.866	2.848	
Pr. W.	16.87	0.542	0.207	0.605	7.86	2.760	4.020	II.
W.	20.27	0.785	0.226	0.629	7.38	2.989	3.934	
Po. W.	16.71	0.623	0.201	0.615	8.59	2.859	3.652	
Pr. W.	17.58	0.610	0.260	0.613	6.60	2.764	3.787	III.
W.	17.96	0.629	0.197	0.671	6.33	2.688	3.816	
Po. W.	17.03	0.636	0.214	0.667	6.69	2.689	3.707	
Pr. W.	16.87	0.552	0.198	0.660	7.20	2.606	3.653	III.
W.	16.92	0.602	0.200	0.692	6.69	2.536	3.737	
Po. W.	16.76	0.638	0.203	0.677	6.90	2.534	3.670	
Pr. W.	14.41	0.630	0.228	0.667	5.70	2.358	3.184	IV.
W.	15.14	0.691	0.220	0.714	4.45	2.555	3.083	
Po. W.	16.41	0.668	0.206	0.686	5.36	2.925	2.938	
Pr. W.	14.18	0.622	0.206	0.667	3.97	2.641	3.284	IV.
W.	15.73	0.618	0.233	0.706	4.58	2.741	3.296	
Po. W.	16.27	0.591	0.248	0.673	4.13	2.848	3.560	
Pr. W.	18.48	0.820	0.225	0.604	9.14	3.142	3.556	II.
W.	20.09	0.909	0.220	0.636	9.94	3.258	3.661	
Po. W.	20.27	0.804	0.209	0.622	9.59	3.234	3.637	
Pr. W.	17.75	0.806	0.268	0.654	8.09	2.871	3.597	III.
W.	19.80	0.789	0.271	0.682	9.39	3.330	3.687	
Po. W.	18.23	0.658	0.269	0.664	8.00	3.162	3.448	
Pr. W.	9.14	0.422	0.198	0.536	5.29	2.038	2.473	I.
W.	9.90	0.390	0.201	0.552	4.92	2.136	2.486	
Po. W.	8.96	0.445	0.154	0.553	4.80	2.049	2.244	

* Pr. W. = Pre Work, W. = Work and Po. W. = Post Work periods.

for pre-work period are the average of the four days preceding work, the work period is the average output of the six days' work, and that of the post-work period the average of the four days after the cessation of work.

Table III.—Averages.

	Series I.			Series II.			Series III.			Series IV.		
	Pre-work.	Work.	Post-work.	Pre-work.	Work.	Post-work.	Pre-work.	Work.	Post-work.	Pre-work.	Work.	Post-work.
Total nitrogen	8.78	10.01	9.05	17.31	19.96	17.76	17.40	18.23	17.34	14.30	15.44	16.34
Ammonia nitrogen	0.426	0.468	0.474	0.706	0.811	0.686	0.656	0.673	0.644	0.626	0.654	0.629
Uric acid nitrogen	0.189	0.198	0.155	0.220	0.229	0.205	0.242	0.223	0.229	0.217	0.226	0.227
Creatinine nitrogen	0.543	0.562	0.552	0.603	0.633	0.620	0.642	0.682	0.669	0.667	0.710	0.679
Total sulphur	0.812	0.853	0.826	1.151	1.223	1.194	1.115	1.140	1.118	0.996	1.059	1.154
Total P_2O_5	2.20	2.33	2.14	3.52	3.47	3.38	3.63	3.75	3.61	3.53	3.19	3.25

The rise in the nitrogen output obtained,* although it is consistently present, is not great and can only account for a very small fraction of the total energy expended. If the rise in nitrogen output during the work and the excess nitrogen output of the post-work period be considered together, the results are very interesting. On diets I and II, which differ from one another solely in the addition of extra protein in the form of cheese in the case of II, giving as a practical result an average nitrogen equilibrium of 8.78 and 17.31 grams respectively, there is, as the result of the work, a comparable rise in the output of nitrogen in the two sets of experiments. The average ratio of the nitrogen intake in I and II is as 1 to 1.97, and the average excess output is as 1 to 2.07.

One might assume that here, as in the general law of nitrogen equilibrium, there was some direct relationship between the intake and output. This, of course, may be the case when conditions remain constant, but, as is shown by the third set of experiments, where the diet only differed from that in the second set by the daily addition of a small amount of meat extractives, although the output of nitrogen in the two pre-periods (*i.e.*, of II and III) differed but little, yet identical amounts of muscle work led to an increase in the output of nitrogen in series III about a third less than that in series II. Instead of 3.10 grams, the average excess output is a little under 1 gram. Again, we might assume that the ingestion of the extractives of muscle tissue had led either to a diminution in the breakdown of nitrogen-containing tissue or that a better retention of nitrogen had taken place. It may be possible that this is the explanation, as Thomas (14), in his work on the biological values of protein,

* The analyses for total nitrogen were carried out three times by three different workers in order to make certain the results were correct. The differences obtained by the three workers in the eleven experiments amounted to under 1 per cent.

found that although ox flesh has only some 88 per cent. of its nitrogen in the form of protein, yet the high biological value of 105 was obtained. Thomas stresses this point, maintaining that extractives seem to play an important rôle in the utilization of protein. But it is equally obvious that the nature of the extractives present must play a determining part, *vide* his experiment with crab muscle. Rubner (15) and Burgi (16) have also devoted some attention to the part played by extractives and are of the opinion that they can be retained in the body when conditions are suitable. As a rule, however, the nitrogen of the extractives appeared to be rapidly excreted. Thompson (17) supported the view that a retention of extractive nitrogen took place. Voltz and Baudrexel (18), on the other hand, stated that the addition of meat extract to a diet did not bring about any increase in the absorption either of the nitrogen-containing or nitrogen-free moieties of the diet. They found, however, that either a slight retention or perhaps a prevention of nitrogen loss could occur. Our experiments, at any rate two out of the three of the extractive series, show that there is quite unmistakably a reduction in the output of nitrogen. Faecal nitrogen output variation does not offer any explanation, as we checked the possibility of this in one experiment and found that muscle work apparently produced little or no alteration in the distribution of nitrogen between faeces and urine. Wilson, working in my (E. P. C.) laboratory, has found that the addition of meat extractives to a nitrogen-free diet seemed to inhibit to some extent the catabolism of tissue protein. There was also some evidence of retention of the meat extractive nitrogen.

But if the fourth series be considered, where one might assume, if anywhere, as the subject was now on a meat diet, that the full value, if any, of the meat extractives as spacers of protein breakdown, or perhaps more correctly of bringing about nitrogen retention, would be demonstrated, it is found that here the excess output of nitrogen as the result of muscle work is, in relation to the average nitrogen intake, the highest of the whole series of experiments. In connection with this increased output there is a point of considerable interest, because the high average nitrogen output is for the most part due to the enhancement of the post-work period figures. The post-work output is in comparison with all the other figures most pronounced. Why, then, if the muscle extractives have, according to the third set of experiments and as previous work would indicate, the power of bringing about retention, is there no evidence of their capacity here? It can hardly be that the result obtained on the administration of the extractives in free form is due to the alteration in water metabolism (retention), presumably due to the salt content. The

problem is one of very considerable interest, and is at present being further investigated.

The output of urea was not determined, as it was considered that the total nitrogen figure, especially if read in conjunction with the data for ammonia, uric acid and creatinine, gave adequate data. A point of some interest here is that the definite rise in the post-work nitrogen output of the fourth series would seem, arguing from past experience, to be for the most part in the form of urea (Shaffer (19), Kocher (20), Dunlop (21) and Campbell (22)).

There is little noteworthy in the figures for ammonia output. A slight but uniform rise in the output of ammonia nitrogen is found in all four series of experiments as the result of muscle work. This is in general agreement with previous work. Shaffer (19) and Campbell (22) both noted that work exercised but little influence on the output of ammonia. Dunlop (21), on the other hand, obtained a rise, and Kocher (20), working with a nitrogen-free diet, got, on the contrary, a definite reduction, which persisted even into the post-work period.

When the uric acid figures are considered, it is very evident that, under our experimental conditions, muscular work has produced but little alteration in the output of uric acid. This was rather unexpected, although the results agree with the observations of Shaffer (19). Rakestraw (23), who carried out careful examinations of the blood after exercise, found that, both with short strenuous exercise and that of a more prolonged type, a small but definite increase in the uric acid content took place, an increase which continued for some time after the cessation of work. Levine (24), who carried out the examination of the blood of Marathon runners before and after the race, also found quite a definite rise in the uric acid content. Kocher (20), in his experiments with the nitrogen-poor diets, found a marked increase in the output of uric acid in the urine as the result of work. Dunlop (21) came to the conclusion that a rise in the output of uric acid takes place if the subject at the time of exercise is in poor condition, whereas if he is well-trained an actual diminution of output may be obtained. Campbell (22) also found a reduction in the output of uric acid in the work periods, both moderate and severe, although, when severe work is compared with the period of rest, there would appear to be some rise in the total purine output. In a recent paper Hartmann (25) found that in all cases muscle work brought about some reduction in uric-acid output. In view of the work of Burian (26) and others, it is highly probable, however, that the effect of work on purine metabolism is completely masked in the 24-hour sample of urine.

As was perhaps to be expected from past experience, muscle work in these experiments had no pronounced effect on the output of creatinine, although inspection shows that there is a consistent, if slight, increase in the amount excreted. Campbell (22) obtained a definite rise in the creatinine output when severe work was done, although moderate work he found to be without influence. The nature of the diet consumed would seem to influence the output, as Kocher (20) got a definite rise in creatinine in both his series of experiments on the low-protein diet, although it must be noted that there was also a rise in one experiment on a nitrogen-rich diet. Rakestraw (23), in his blood examinations, found a slight rise both in the course of his short- and his long-work experiments.

The partial examination of the nitrogen partition in the urine has, then, disclosed but little. The outstanding fact is the slight but constant increase in the output of total nitrogen, which would seem in the main to be due to increase in urea output which results from the performance of muscle work.

If the increased output of nitrogen which results from the performance of muscular work brings about a true increase in the catabolism of protein, then there should also be evidence of this catabolism in the output of sulphur. That the sulphur output rises *pari passu* with the nitrogen is very evident from the consideration of Tables II and III, and from that of the sulphur-nitrogen ratios, Table IV.

Table IV.—Sulphur-Nitrogen Ratios.

A = of Total Outputs.

Series I.			Series II.			Series III.			Series IV.		
Pre-work.	Work.	Post-work.	Pre-work.	Work.	Post-work.	Pre-work.	Work.	Post-work.	Pre-work.	Work.	Post-work.
Sulphur.	Nitrogen.	Sulphur.	Nitrogen.	Sulphur.	Nitrogen.	Sulphur.	Nitrogen.	Sulphur.	Nitrogen.	Sulphur.	Nitrogen.
1 : 10.8	1 : 11.7	1 : 10.95	1 : 15.0	1 : 16.3	1 : 15.0	1 : 15.6	1 : 16.0	1 : 15.6	1 : 14.3	1 : 14.6	1 : 14.2

B = of Excess Outputs.

Sulphur. Nitrogen.	Sulphur. Nitrogen.	Sulphur. Nitrogen.	Sulphur. Nitrogen.
1 : 27.5	1 : 26.7	1 : 28.8	1 : 14.8

It is interesting to note that although the intakes of sulphur did not bear the same relation to one another as did the total nitrogens in Series I and II, yet when the excess outputs, which result from the work, are considered it is found that they bear practically the same ratio to one another as did the excess output of nitrogen, viz., 1 : 2·13 in the case of sulphur and 1 : 2·07 in the case of the nitrogen.

Further, it will also be observed that, just as is the case with the nitrogen, in series III the total excess output of sulphur is the lowest of all, and, again, as occurred in the nitrogen excretion, the output of sulphur in series IV is the most pronounced of all, agreeing with the nitrogen output even in being definitely higher in the post-work period.

This remarkable parallelism in the outputs of sulphur and nitrogen affords very striking evidence that the effect of muscle activity is to increase the catabolism of some nitrogen-sulphur-rich tissue, presumably muscle. The only difficulty about accepting muscle as the mother substance is that, with the exception of series IV, the sulphur-nitrogen ratios of the excess outputs are divergent from that of the average composition of muscle. The sulphur-nitrogen ratio of muscle is generally accepted as being about 1 : 14, whereas the ratios for the excess outputs in the four series of the present experiments are :—series I, 1 : 27·5; II, 1 : 26·7; III, 1 : 28·8; IV, 1 : 14·8. In series IV the value for the excess output differs but little from the ratio value of the total output. In the case of the other three series the excess output values differ very markedly from the total values. When the sulphur-nitrogen ratios of the average total outputs for the pre-work, work and post-work periods are examined (Table IV), it is seen that they certainly demonstrate that, so far as the ratios are concerned, the post-work period values have returned, to all intents and purposes, to pre-work value, and that, in each case, as the result of the work there is a small but definite fall in the ratio.

A possible and even probable explanation of the curiously low excess output sulphur-nitrogen ratios found is that, simultaneously with the undoubted increase in catabolism induced by muscle activity, there is a definite stimulation of anabolism. Wilson (27) has produced a certain amount of evidence that storage or retention of sulphur in the organism readily takes place, hence, if a retention of sulphur results for the purpose of building up new body (muscle) tissue, as indubitably occurs in the eutrophy of Virchow (see also Bornstein (28)), then the abnormally low ratios of the excess outputs are explicable. The sulphur-nitrogen ratios of the excess output in series IV may be regarded as substantiating this hypothesis in that, as the protein fed had a sulphur-nitrogen

composition suitable either for the repair or the building up of new muscle tissue, no preferential retention of sulphur would be required, and hence the sulphur-nitrogen ratio of the excess output on the meat diet would retain its normal level.

The outputs of chloride and phosphorus were also followed, but the results are too irregular to allow of any interpretation or deductions. This agrees with the results obtained by Kaup (32).

PART II.

Gaseous Metabolism.

Throughout the whole series of experiments the influence of the muscle work on the gaseous metabolism was also determined. Owing to technical difficulties it was impossible to carry out the periods of work under strictly basal conditions. In order, then, to make our determinations of the net changes due to the performance of muscle work reliable, the resting metabolism of the subject was determined daily at a definite hour immediately preceding the work experiment. The subject took his breakfast between 7 and 7.30 a.m., and about 10.30 a.m. he lay down on the metabolism bed at complete rest for 30 to 40 minutes. Immediately after the collection of the resting metabolism samples the subject carried out the hour's work on the ergometer. During the period of work which, as already mentioned, was of approximately constant amount and at approximately constant rate, three collections of his expired air were made after (1) 20–25 minutes, (2) 40 minutes and (3) 55 minutes by means of the ordinary Douglas bag, using a mouthpiece. The subject from long experience, (months), had no difficulties or discomfort with either breathing or wearing the apparatus. During the course of the experiment his respiration and pulse rates were observed thrice. The data regarding these physical observations as regards the heart and circulation are dealt with in part in a paper by Gillespie, Gibson and Murray (29). In a certain number of instances observations were carried out by means of a rectal thermometer on the course of the body temperature, but not in sufficient number to permit of any conclusions being drawn.

In order to determine the actual net cost of the work performed the resting metabolism (per minute) of the day was deducted from the total metabolism (per minute) of the work period. The analyses of the air samples were naturally always done in duplicate.

As regards the resting values for the oxygen intake, the mean for all experiments was 275.5 c.c. per minute—a good average value under our conditions.

When the individual values of the resting oxygen consumption were averaged for each series, it will be seen from the following Table V that except for series I there is seemingly a rough relation between the level of the protein intake and the oxygen consumption.

Table V.—Respiratory Quotients and Oxygen Utilization in cubic centimetres per minute.

Series	Series I.		Series II.		Series III.		Series IV.	
	Rest.	Work.	Rest.	Work.	Rest.	Work.	Rest.	Work.
Mean R.Q.	0.86	0.92	0.86	0.91	0.88	1.00	0.85	1.00+
Mean Oxygen p.m.	270	1,041	284	1,073	277	939	271	898

Average resting oxygen = 275.5; average R.Q. resting = 0.863.

Brief reference may be made here to the alterations which took place in the respiratory quotient, both during the rest periods and as the result of work. The average R.Q. for all rest periods was 0.863, the averages for the individual series, in spite of the differences in the nature of the diet, being wonderfully close. When the work R.Qs. are considered it will be noted that the four series fall into two groups, the meat-free series I and II being lower than series III and IV, which contained either meat extractives or meat. All four, however, agree in being higher than the resting values. Although others deny that it takes place, both Benedict and Cathcart (30), and Douglas and his co-workers (31), have previously observed this rise in the R.Q. value as the result of work. Presumably it indicates a preferential combustion of carbohydrate. As the subject was a well-trained subject, in the pink of condition and well accustomed to the apparatus, it is difficult to believe that the result is due to some technical (in its broadest sense) flaw. Of the three determinations which were made in the course of the period of work it may be said that there was a very distinct tendency for the R.Q. of the first determination to be highest. No solution can be offered for the consistently high values of the work periods in series III and IV. Benedict and Cathcart (30) obtained high respiratory quotients as the result of muscle work even with their subject in the post-absorptive condition. See also the work of Furusawa (33).

The effect of muscle work on the gaseous metabolism is given both in terms of oxygen consumption and calories per minute and per kilogrammetre external

work done. In view of the fact that the determination in terms of calories is based on the respiratory quotient, the magnitude of which is at the mercy of the carbon dioxide content of the expired air, and hence is liable to considerable error, more stress will be laid, as on the sounder and more reliable base, on the consumption of oxygen, especially as in these experiments there never was any question of taxing the organism and so upsetting the oxygen balance. Moreover, it is contended that the values obtained are approximately true values, in that they are based on three separate determinations during the course of the hour's work, the first collection not being made until 20 to 25 minutes after the start of work, when it may be assumed, with a highly trained and well practised subject, that a state of equilibrium or adjustment to the immediate needs had been attained.

Table VI shows very clearly that the average oxygen intake per minute is definitely reduced in the series III and IV, *i.e.*, in the series in which meat extractives and meat were used. The results as a whole would have been even more striking if it had not been for the definite fall in the oxygen consumption which took place in the third experiment on the basal diet I. The only explanation which can be offered for this very definite discrepancy, and it may be remarked that the values obtained in all the determinations of which the averages alone are here given showed very good agreement, is that this experiment followed immediately on an experiment made with meat extractives. Possibly the slightly higher oxygen consumption found with diet II as compared with I is due to the increased content of protein, although, if protein is to be considered the sole cause, it is difficult to reconcile with the consistently lower utilization of oxygen in diet III with the same, or indeed slightly higher, nitrogen intake.

Naturally the question arises whether these variations in value are to be considered significant or not. In order to determine this recourse was had to statistical treatment of the data, not, be it stated, with the view of lending a fictitious air of accuracy by the parade of a mathematical nicety, but because such a method of treatment is at present the only possible avenue of approach. The values selected were the standard deviation (SD or σ), the constant which measures in absolute terms the degree of dispersion of the data, the coefficient of variation (CV) which is the expression of the standard deviation as a percentage of the mean and the probable error (PM) of the mean. Finally, in order to determine whether the values obtained are significant, *i.e.*, whether the differences found point to a real difference existing between the varying data obtained, and hence justify the conclusion that the variation is significant

Table VI.

No. of Experiment.	Diet.	Revolutions per Hour.	Average Revolutions per Hour.	No. of Observations.	Average kgm. per Minute.	Average (over all) kgm. per Minute.	Average Net O_2 Intake in c.c. per Minute.	Average (over all) O_2 per Minute.	No. of Observations.	Average net Cals. per Minute.	Average (over all) Cals. per Minute.	No. of Observations.	Average net O_2 in c.c. per kgm.	Average (over all) O_2 per kgm.	No. of Observations.	Average net gr. cals. per kgm.	Average (over all) gr. cals. per kgm.	No. of Observations.	Average net Eff. efficiency per cent.	Average (over all) Efficiency per cent.	No. of Observations.
I	I	25,209	25,150	17	424.9	422.7	1130.0	1041.3	48	5.57	5.20	48	2.66	2.47	48	12.93	12.28	48	18.0	19.1	48
II	I	25,208			422.7		1061.8			5.29			2.51			12.51			18.8		
III	I	25,043			418.8		961.5			4.86			2.29			11.61			20.2		
IV	II	25,187	25,098	18	425.5	423.0	1124.3	1072.6	54	5.60	5.36	54	2.64	2.53	54	13.13	12.65	54	17.8	18.6	54
V	II	25,013			423.0		1080.6			5.38			2.55			12.73			18.4		
VI	II	25,060			421.2		1005.7			5.06			2.39			12.03			19.5		
VII	III	25,111	25,128	18	422.8	422.7	960.2	938.6	51	4.87	4.77	53	2.26	2.22	51	11.50	11.26	51	20.4	20.9	51
VIII	III	25,210			422.9		900.8			4.58			2.12			10.79			21.7		
IX	III	25,065			422.4		955.7			4.86			2.26			11.51			20.4		
X	IV	25,066	25,125	11	417.6	418.4	889.9	898.1	33	4.53	4.57	33	2.13	2.15	32	10.85	10.94	32	21.6	21.5	32
XI	IV	25,198			419.2		907.9			4.62			2.16			11.03			21.3		

and not merely due to chance, arising from inadequate sampling, the formula $0.6745 \sqrt{\frac{\sigma^2}{n_1} + \frac{\sigma^2}{n_2}}$, where 0.6745 is a constant, n_1 and n_2 the numbers of experiments compared, and σ the standard deviations of the respective sets of experiments, was utilized. It is customary to maintain that any difference greater than three or more times the probable error is almost certainly, or even certainly, significant.

Considering the fact that these results are derived from eleven separate experiments, each of six days' duration, spread over a year, totalling some 200 duplicate analyses, with all the opportunities of chance variation from innumerable sources, it will be noted that all the values obtained indicate a very satisfactory agreement and particularly a close adherence of the subject to the detail of the experiment and the elimination of most of the controllable limiting factors. But the really important question is whether or no the differences

Table VII.—Statistical Data. Net Oxygen Utilization per minute in cubic centimetres.

	Series I.	Series II.	Series III.	Series IV.
No. of experiments	48	54	53	33
Standard deviation	97.752	66.900	51.74	40.120
Coefficient of variation	9.387	6.237	5.514	4.467
Probable error of mean	9.517	6.192	4.794	4.711
Arithmetic mean	1041.25	1072.63	938.58	898.12

By use of formula $0.6745 \sqrt{\frac{\sigma^2}{n_1} + \frac{\sigma^2}{n_2}}$ ratios found are:—

	Difference of Means.	Probable Error of Difference of Means	Ratio.
II and I	31.38	11.33	2.77 : 1
I and III	102.67	10.66	9.63 : 1
I and IV	143.13	10.62	13.57 : 1
III and IV	40.46	6.72	6.02 : 1

The chances of reaching the same results on mere random sampling:—

II and I	1 to 15.
I and III	1 to >55,000,000.
I and IV	Odds infinite.
III and IV	1 to 20,324.

observed are to be regarded as significant. When analysed by means of the formula given above it will be seen (Table VII) that no significance is to be attached, the ratio being less than 3 : 1, to the small difference found between the oxygen intakes of series I and II. But taking the worst value from a

comparative point of view, the lower oxygen intake of series I, for comparison with the still lower oxygen intakes of series III and IV, it is obvious that there would seem to be real significance between I and III, as the ratio is over 9 : 1, and between I and IV, where it rises to over 13 : 1.

These differences are even more clearly displayed if the still more regular series of results obtained by a comparison of the oxygen utilization per kilogrammetre of work done is examined. These figures must obviously be somewhat less discordant than the simple net oxygen consumption results, as they are free from the certain amount of distortion due to the unavoidable variations in the amount of external work performed per minute. The results of the determination of these values with the appropriate statistical analyses are found in Tables VI and VIII.

Table VIII.—Statistical Data. Net Oxygen Utilization per kilogrammetre.

	Series I.	Series II.	Series III.	Series IV.
No. of experiments	48	54	51	32
Standard deviation	0.2225	0.1580	0.1228	0.0964
Coefficient variation	9.01	6.24	5.54	4.49
Probable error of mean	0.0217	0.0145	0.0116	0.0115
Arithmetic mean	2.47	2.53	2.22	2.15

By use of formula $0.6745 \sqrt{\frac{\sigma^2}{n_1} + \frac{\sigma^2}{n_2}}$ ratios found are :—

	Difference of Means.	Probable Error of Difference of Means.	Ratio.
II and I	0.064	0.026	2.46 : 1
I and III	0.252	0.024	10.26 : 1
I and IV	0.322	0.024	13.14 : 1
III and IV	0.070	0.016	4.29 : 1
Real mean (I and II) and IV	0.356	0.017	20.60 : 1

It will be observed that, naturally, the figures follow the same course as in the net utilization and bring out clearly again the consistent fall in the consumption of oxygen in series III and IV. When they are considered as regards their significance, again there would appear to be none between I and II, whereas the differences between I and III and I and IV appear to be definitely significant. When III and IV are contrasted the ratio is over 4 : 1, therefore possibly significant, and if the mean of I and II taken together be contrasted with that of IV the very high ratio of 20.6 : 1 is obtained. Although the number of analyses compared is relatively small, in view of the uniformity of the conditions and the

accuracy of the results, it is considered that weight may be laid on the statistical treatment and that accordingly it may be concluded that the presence of meat or meat extractives in a diet used for the performance of muscular work does bring about a material alteration in the oxygen intake.

Naturally, as the calorie values when determined indirectly must for the most part depend on the value of the oxygen intake they, obviously, must follow the course of these oxygen values. Table VI shows clearly that, if one desires to consider the data in terms of calories, then the cost per kilogrammetre in gram calories is definitely lowest on a meat diet. Hence it follows that if the work done is considered with regard to its "efficiency" of performance (see Table VI) the net efficiency (effectivity) is highest on the meat and meat extractive diets. The efficiencies* (effectivities), if they are of any real importance at all, are on the whole fairly constant and would indicate that the particular type of arm movement, a simple to-and-fro one, used in these experiments was not a particularly economical one.

In addition to the foregoing experiments, a number of other experiments were carried out, and brief reference will be made here to the two in which an exclusively fat diet was used.

The subject restricted his diet for three days† to approximately 360 grams of olive oil *per diem*, taken in three doses. He carried out the routine experimental work as in the preceding experiments. In the first oil series the amount of work done averaged only 136.25 kilogrammetres per minute, but in the second it was raised to the usual amount and averaged 429 kgm. per minute. The results obtained are summarized in Table IX.

Again the rise in the respiratory quotient during the work period in both experiments is found. The pre-work resting values in both series are typical fat-combustion ratios. The work value in series F. II gives a somewhat higher

* In the Industrial Fatigue Research Board (Medical Research Council) Report, No. 29, E. P. C. suggested that the term efficiency, which has a well-defined or, according to the conditions, a number of well-defined meanings in engineering science, should not be applied to the human organism but should be replaced by some such general term as effectivity, which would express the results obtained without suggesting that the relations are fundamentally thermal in nature.

† This is the maximum period for an exclusively fat diet which repeated experiment, with several subjects, including both authors, in this laboratory, has shown to be compatible with reasonable comfort. After the third day nausea is so extreme that it is impossible to ingest the necessary amount of fat. Long experience has taught us that *pure* olive oil is the easiest and least nauseating fat to take. It is bland and is particularly well utilized in the gastro-intestinal canal. The addition of a very small amount of potassium carbonate solution permits, if so desired, of emulsification, in order to form a cream-like material.

Table IX.—Experiments with a Diet of Olive Oil (Work).

						F. I.	F. II.
Kilogrammetres per minute	136.25	429.1
Average resting R.Q.	0.75	0.75
Average work R.Q.	0.81	0.88
Average net oxygen cubic centimetre per minute rest	268	250
Average net oxygen cubic centimetre per minute work	367	1,008
Average net oxygen cubic centimetre per kgm.	2.70	2.35

Statistical Data				Mixed Diet.	F. I.	F. II.
Arithmetic mean, O ₂ per kgm.				2.64	2.697	2.349
S.D.				0.2554	0.1908	0.06725
Probable error of mean				0.0861	0.0525	0.01512

value than series F. I., but neither indicates that carbohydrate contributed exclusively for the yield of the necessary energy for work. It is always possible, of course, that in such experiments, owing to the drastic alteration in intermediate metabolism, the R.Q.—which, after all, is but a ratio dependent on many and mostly unknown components—may not give a true index of the work changes pure and simple. Each work period extended over one hour, and three collections of expired air were made and analyzed in duplicate. In the first series, as it was thought that the presence of acetone in the expired air might vitiate the analyses, a second sample of air washed with acid water was also analyzed. The results obtained differed but little from those done by the ordinary method.

In spite of the fact that, chemically at least, the breakdown of fat into combustible fragments requires a considerable intake of oxygen, there is but little evidence of this demand if the net oxygen requirement per kilogrammetre of work calculated from the resting values is considered. The average value obtained, 2.70 c.c. in F. I., is very slightly above the average, 2.64 c.c., of those found for this subject on an ordinary mixed diet, and in F. II., 2.35 c.c., it is even below the average. Further, if the attempt be made to calculate the absolute net demand of energy for the production of external work, by determining the oxygen consumption per wheel revolution per minute, whilst the subject causes the wheel to rotate without load (the friction is but slight (13)), and deducting this value from the oxygen consumption per wheel revolution per minute with a known load, and then dividing the net consumption per revolution per minute by the actual amount of external work done per minute

in kilogrammetres, it is found (Table X) that in the control experiment on an ordinary mixed diet the average oxygen demand is 0.035 c.c. per kilogrammetre (four sets of analyses), and 0.036 c.c. (six sets of analyses) on the fat diet—an astonishingly good agreement.

Table X.—Absolute net Oxygen Utilisation in cubic centimetres per kilogrammetre (*i.e.* Load less No Load Values).

Diet.	Mixed.	Carbohydrate.	Fat.
Oxygen values in cubic centimetres (mean)	0.03575	0.03125	0.03566
S.D.	0.00327	0.00512	0.00398
Probable error of mean	0.00110	0.00172	0.00109

In both sets of fat experiments the fat diet was followed by the ingestion of carbohydrate, in series F. I by two days of an exclusively carbohydrate diet (tapioca and sugar), and in series F. II by one day of wheatmeal and sugar. In the first series the average net oxygen utilization per kilogrammetre, calculated as usual by deducting the ordinary resting consumption of oxygen, for the first day was 2.03 c.c. and for the second day 2.63 c.c., whereas in the second series the average for the first and only day was 2.26 c.c., *i.e.*, the change from a fat to a carbohydrate diet brings about a slight reduction in the cost in terms of oxygen per kilogrammetre. In the first series the no-load experiments were also done, and when the calculation is made as above, it is found that the absolute oxygen requirement per kilogrammetre on the first carbohydrate day was 0.028 c.c. and on the second day 0.034 c.c., giving an average of 0.031 c.c. for both days, a value slightly below those found both for ordinary and pure-fat diets. (See Table X.)

In Table XI there is given the record of the influence of the giving of an oil diet, at rest and at work, on the output of the urinary constituents. Again

Table XI.—Experiments on a Diet of Olive Oil. Excretion of Nitrogen and Sulphur in grams.

Day	No Work Experiment.			Work Experiment (F. II).		
	1	2	3	1	2	3
Total nitrogen	8.4	11.8	12.4	9.5	14.2	13.7
Ammonia nitrogen	0.47	0.71	0.84	0.44	0.61	0.84
Uric acid nitrogen	0.27	0.29	0.50	0.13	0.14	0.22
Creatinine nitrogen	0.47	0.51	0.59	0.57	0.77	0.63
Total sulphur	0.688	0.910	0.936	0.826	0.970	0.942

it will be noted that the rise in N. output characteristic of a fat diet is exaggerated by muscle work. As in the other experiments, the rise in nitrogen output is accompanied by a rise in the output of sulphur.

Summary.

The results obtained may be cautiously and briefly summarized as follows :—

1. Muscular work brings about a definite, although small, rise in the output of nitrogen.
2. It also produces a parallel rise in the output of sulphur.
3. There also results small, rather insignificant, changes in the distribution of nitrogen in the urine.
4. The different types of diet employed influence the course of the gaseous metabolism during work.
5. Under the conditions of the present experiments the differences in the oxygen demand during work on diets which contain meat and those which are meat-free are definitely significant.
6. The oxygen consumption per kilogrammetre of external work is not materially different when the subject is on an ordinary mixed diet or an exclusively fat diet, but there is, perhaps, some evidence that when on an exclusively carbohydrate diet the oxygen demand is slightly lowered.

Our thanks are due to Dr. Wishart and Miss Bedale for their assistance in the conduct of some of the experiments, and to Dr. S. Dawson for his advice in the statistical treatment.

The expenses were defrayed in part by a grant from the Government Grant Committee of the Royal Society and in part by the Carnegie Trust.

LITERATURE.

- (1) Chittenden, 'Physiological Economy in Nutrition,' London (1905).
- (2) Hindhede, 'Protein and Nutrition,' London (1913).
- (3) McCay, 'Protein Element in Nutrition,' London (1912).
- (4) Campbell, 'Biochem. Journ.,' vol. 13, p. 239 (1919).
- (5) Melville, 'Brit. Med. Journ.,' vol. 2, p. 1337 (1910).
- (6) Rubner, 'Volksernährungsfragen,' Leipzig (1908).
- (7) Rubner, 'Die Gesetze des Energieverbrauchs bei der Ernährung,' Leipzig (1902).
- (8) Lusk, 'Medicine,' vol. 1, p. 311 (1922).
- (9) Benedict and Carpenter, 'Carnegie Instit. Publication No. 126' (1910).

- (10) Cathcart and Orr, 'Energy Expenditure of the Recruit in Training, London' (1919).
 - (11) Benedict, Miles, Roth and Smith, 'Carnegie Instit. Publication No. 280' (1919).
 - (12) Cathcart, 'Physiological Reviews,' vol. 5 (1925).
 - (13) Cathcart, Wishart and McCall, 'Jour. Physiol.,' vol. 58, p. 92 (1923).
 - (14) Thomas, 'Arch. f. Physiol.,' p. 219 (1909).
 - (15) Rubner, 'Arch. f. Hyg.,' vol. 51, p. 19 (1904).
 - (16) Burgi, 'Arch. f. Hyg.,' vol. 51, p. 1 (1904).
 - (17) Thompson, 'Proc. Brit. Assoc.,' 1910.
 - (18) Voltz and Baudrexel, 'Arch. f. ges. Physiol.,' vol. 138, p. 275 (1911).
 - (19) Shaffer, 'Amer. Jour. Physiol.,' vol. 22, p. 445 (1908).
 - (20) Kocher, 'D. Arch. f. klin. Med.,' vol. 115, p. 82 (1914).
 - (21) Dunlop, Paton, Stockman and Macadam, 'Jour. Physiol.,' vol. 22, p. 68 (1897).
 - (22) Campbell and Webster, 'Biochem. Jour.,' vol. 15, p. 660 (1921); vol. 16, p. 106 (1922).
 - (23) Rakestraw, 'Jour. Biol. Chem.,' vol. 47, p. 565 (1921).
 - (24) Levine, Gordon, Derich, 'Jour. Amer. Med. Assoc.,' vol. 82, p. 1778 (1924); also
'Arch. Int. Med.,' vol. 33, p. 425 (1924).
 - (25) Hartmann, 'Arch. f. ges. Physiol.,' vol. 204, p. 613 (1924).
 - (26) Burian, 'Med. Klinik,' No. 6 (1905).
 - (27) Wilson, 'Biochem. Jour.,' vol. 19, p. 322 (1925).
 - (28) Bornstein, 'Arch. f. ges. Physiol.,' vol. 83, p. 540 (1901).
 - (29) Gillespie, Gibson and Murray, 'Heart,' vol. 12, p. 1 (1925).
 - (30) Benedict and Cathcart, 'Carnegie Instit. Publication No. 187' (1913).
 - (31) Campbell, Douglas and Hobson, 'Phil. Trans.,' B, vol. 210, p. 1 (1920).
 - (32) Kaup, 'Zeit. f. Biol.,' vol. 43, p. 221 (1902).
 - (33) Furusawa, 'Roy. Soc. Proc.,' B, vol. 98, p. 65 (1925).
-

The Life-Cycle of the Nodule Organism, Bacillus Radicicola
(BEIJ.), in Soil and its Relation to the Infection of the Host
Plant.

By H. G. THORNTON, B.A., and N. GANGULEE. Ph.D.

(Communicated by Sir John Russell, F.R.S.—Received January 7, 1926.)

A. INTRODUCTION.

The existence of changes in the form of *Bacillus radicicola* has been known since Beijerinck (2) first isolated it in 1888 from leguminous plant nodules. He observed the motile "swarmer" stage as well as the branching forms, whose nature was already the subject of controversy. About the same time the development of straight-rod forms of the organism was described by Prazmowski (14). Numerous writers have since observed the existence of the organism in the three conditions of straight rods, branching rods and cocci (for references, see Löhnis, 1921 (10)). In 1916 Löhnis and Smith (11) claimed that the various forms constituted a definite life-cycle through which the organism normally passes, and this cycle, as seen in cultures, was carefully described in 1919 by Bewley and Hutchinson (3). In a vigorous young culture, the predominating form of the organism is a short, evenly staining rod (fig. 1). These rods soon undergo a change in internal structure, the staining material becoming segregated into bands crossing the cell. During this banded stage the cells frequently become swollen, distorted, and branched, the so-called "bacteroids" (Brunchorst (4)), but this irregularity of form is not an essential part of the life-cycle, but would appear to be a response to conditions of the environment (Buchanan, 1909) (5). The banded cells give rise to the cocci by further condensation of the bands.

The origin of the cocci within the mother-cell was described and illustrated in 1891 by Morck (12), who was the first to appreciate the relation of the internal structure of the cell to the life-history of the organism. The cocci are usually released in a non-motile condition, and afterwards develop flagella, becoming actively motile, the "swarmers" of Beijerinck (2). Under certain conditions, however, the cocci develop flagella while still enclosed within the mother-cell. This condition has been described by Greig-Smith (8) and the observation confirmed by one of the present authors (7). The cocci eventually become elongated and thus pass into the unbanded rod stage. The flagella, which are

developed on the cocci, persist after this elongation, but are soon lost: the rods then become non-motile. The development of motility in a culture is thus intimately associated with the appearance of the coccus stage.



THE LIFE CYCLE OF *BACILLUS RADICICOLA*

FIG. 1.

In 1922 Wallin (15) observed the existence of various stages of the organism within the tissues of the nodule. The object of the present work was to discover whether the nodule organism undergoes a similar cycle of changes in soil, and, if so, whether the incidence of the motile coccus stage is associated with a migration of the bacteria through the soil.

B. TECHNIQUE.

(1) *Method of studying the Life-Cycle in Soil.*

The nodule organism that infects lucerne (*Medicago sativa* L.) was used throughout the work. The strain was originally supplied by the Statens Planteavlslaboratorium, Lyngby, Copenhagen, and its ability to produce nodules was frequently tested. The cultures were grown on an agar medium containing mineral salts, saccharose, and extract of lucerne roots, and were incubated at 25° C. for 10 days before use.

Petri dishes, 6 inches in diameter, containing 200 grs. of a mixture of Rothamsted soil (34 per cent.) and sand (66 per cent.) were sterilised at 15 pounds pressure for 30 minutes, and the water content was made up to

17 to 18 per cent. on an air-dry basis. An agar slope culture was filled to the top of the slope with sterile inoculating fluid, the bacterial growth scraped off with a sterile platinum loop and, when the sediment had settled, 1 c.c. of the suspension was removed in a sterile pipette and evenly distributed over the petri dish of soil. The soil was then thoroughly mixed with a sterile spatula and incubated at 25° C.

On each occasion of sampling three cores were removed from different parts of the petri dish by means of a sterile cork borer. These were well mixed in a watch-glass containing 1 c.c. of sterile distilled water, the sample was triturated with a small sterile rubber pestle and, after standing for 10 minutes, two loopfuls of the surface fluid were spread over a clean slide and the film dried at 45° C. in an oven.

In staining the films a modification of Winogradsky's method (16) was used. The dried film was fixed with absolute alcohol and flooded with phenol-erythrosine (1 gram of erythrosine dissolved in 100 c.c. of 5 per cent. phenol), which was allowed to act for 10 minutes. After washing in tap water the film was re-stained with 2.5 per cent. aqueous erythrosine for another 10 minutes. Duplicate preparations were made from each sample, and, from these, the percentages of unbanded rods, banded rods, and cocci were determined for each slide by counting the numbers of each stage in five random microscope fields.

(2) *Test of Accuracy of the Sampling and Microtechnique.*

Table I.*—Showing the Observed and Expected Numbers of Bacterial Forms.

Samples.	Total number of cells counted.	Number of cocci.		Number of unbanded rods.		Number of banded rods.	
		Observed.	Expected.	Observed.	Expected.	Observed.	Expected.
A	277	13	13.66	257	255.36	7	7.98
B	337	18	16.61	308	310.67	11	9.72
C	290	16	14.30	267	267.34	7	8.36
D	171	6	8.43	159	157.64	6	4.93
Mean percentage		4.93	—	92.19	—	2.88	—
Percentage standard error		0.66	—	0.82	—	0.51	—

* The authors are indebted to Mr. R. A. Fisher for his advice, and, in particular, for making a statistical test of the data given in Table I.

The accuracy of the sampling and microtechnique was tested in four simultaneous samples, taken during the course of experiment 1. From the counts thence obtained the mean percentage of each cell-type was calculated for the whole set of samples. The expected numbers of each type in the individual samples were then obtained by taking the mean percentage of the total cells counted in that sample. Table I shows the expected numbers with the actual numbers observed. The variation between individual samples is not greater than necessarily follows from the random distribution of organisms in the soil.

C. LIFE OF THE ORGANISM IN STERILE SOIL.

Experiment I.

In the preliminary study of the organism growing in sterile soil, a suspension in distilled water was used to inoculate the soil as described above. Samples of soil were examined immediately after inoculation, and, thereafter, at hourly intervals for 27 hours and then at daily intervals for six days.

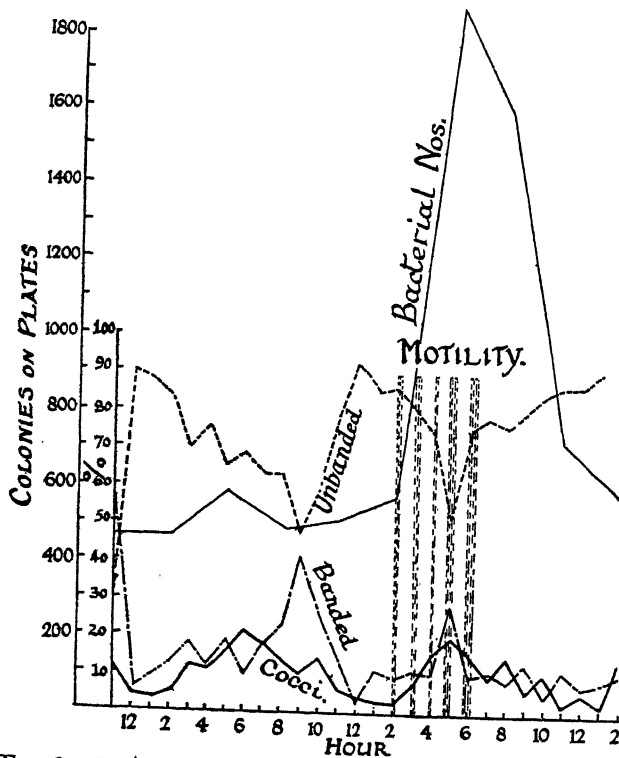


FIG. 2.—*Bacillus radicum*. Life-cycle changes in sterile soil.

(1) *The Life-Cycle Changes.*

In any sample examined, all the three principal cell-types occur, but their proportions vary at different times. The study of the life-cycle is therefore a quantitative problem, it being necessary to follow the changes in the relative numbers of these stages. The changing percentages of each cell-type during the first 27 hours is illustrated in fig. 2. Within this period the population passed through two complete cycles of changes, each commencing with an increase in the proportion of unbanded rods and followed by a rise in the proportion of cocci and banded rods. In both cycles the maximum percentage of cocci is reached as soon as, or before, that of the banded rods from which they arise. This feature is also seen in later experiments (figs. 3, 4, 5, 6, 7), and may be due to a change in the relative rates at which cocci are formed and at which they are released from the rods. The regularity of the changes here observed was shown in a second soil culture set up in a similar manner, from which samples were examined every two hours for 14 hours. The percentages of each cell-type found in this culture are plotted in fig. 3, together with the percentages found at equivalent times in experiment 1.

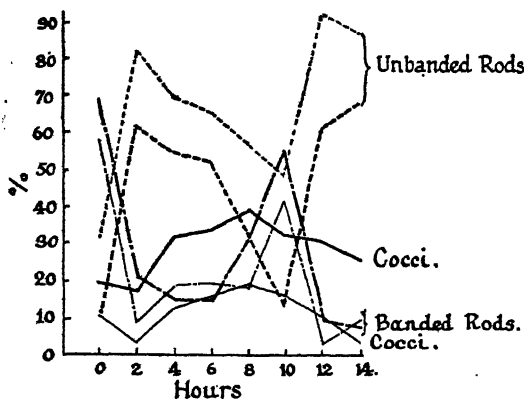


FIG. 3.—Life-cycle changes found in two experiments.

(2) *Multiplication.*

Bacillus radicicola multiplies in two ways, by binary fission of the rod stage and by multiple fission, the banded rods breaking up into cocci. Plate counts made during the course of the experiment indicate that the latter method is the more important, for during periods of maxima of the unbanded rods the numbers show no increase, but increases occur on both occasions when the cocci reach their maxima.

(3) *Motility.*

To study the relation between the life-cycle and the occurrence of motile forms in the soil, a drop of soil suspension was examined in a fresh state at each time of sampling and the presence of motile forms was noted. The vertical broken lines in fig. 2 show the times when motile cells were observed. Motile forms were not evident within the first 12 hours, perhaps owing to the small total number of cells in the culture. The greatest number of motile cells was found in the samples taken at 5 a.m., when the cocci had reached their second maximum, and at 6 a.m., when the relative numbers of cocci were falling off and being replaced by unbanded rods. This is in accordance with the fact observed in liquid cultures, that flagella are developed on the cocci and persist for some time after these lengthen to form the unbanded rods.

(4) *Later Course of the Life-Cycle.*

The subsequent history of the soil culture was followed in experiment 1 by taking samples at daily intervals for six days, and the percentages of each cell-type during this period are shown in fig. 4, where also the earlier history of the culture, already described, is summarised by plotting points selected at six-hourly intervals. In the first 24 hours the cocci never exceed 25 per cent. of the population, but a big increase subsequently occurs, so that by the third day about 70 per cent. of the cells are in this stage.

The chief features of the life-cycle during the first six days were confirmed by a separate experiment carried out under conditions similar to experiment 1, during which samples were examined at daily intervals. In fig. 4 the results of the two experiments are plotted together. The fact that the changes in relative numbers of each cell type, both at hourly intervals during the first 12 hours (fig. 3) and at daily intervals during six days (fig. 4), are so closely reproduced in entirely separate experiments shows that a regular cycle occurs in the soil, the principal features of which are constant in a given environment. It was important to determine whether this cycle was inherent and unalterable, or whether it could be modified by changes in the environment.

D. INFLUENCE OF THE INOCULATING FLUID IN MODIFYING THE LIFE-CYCLE.

Bewley and Hutchinson (3) observed that in cultures of *B. radicola* a plentiful supply of calcium phosphate in the medium stimulated the appearance of the motile coccus stage. To see whether the nature of the suspending fluid used in inoculating soil influenced the life-cycle in the soil, experiments were

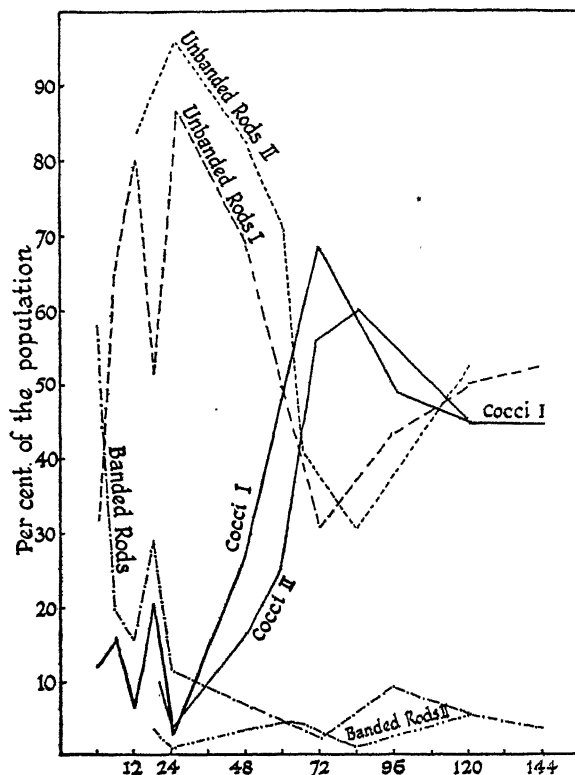


FIG. 4.—Later course of the Life-cycle of *Bacillus radicicola* in soil inoculated with a suspension in water. (Two experiments.)

conducted in a manner similar to experiment 1, save that the following fluids were used to make the inoculating suspension :—

A. Milk.

B. 0·1 per cent. solution of di-acid calcium phosphate ($\text{CaH}_4(\text{PO}_4)_2 + 4\text{H}_2\text{O}$) in water.

C. 0·1 per cent. solution of di-acid calcium phosphate in milk.

Samples of the soil cultures were examined at daily intervals for six days. The changing percentages of cocci, banded and unbanded rods, are shown in figs. 5, 6 and 7, which should be compared with experiment 1 (fig. 4),*where the inoculum was suspended in distilled water. In all these experiments a high percentage of unbanded rods at the twenty-fourth hour gives place to a rise, first in the proportion of cocci, and secondly in that of banded rods. The nature of the inoculating fluid, however, strikingly affects the time and degree of coccus formation (fig. 8).

With distilled water as the inoculating fluid, the percentage of cocci increases but slowly for 48 hours and reaches a maximum at the third day, then falling

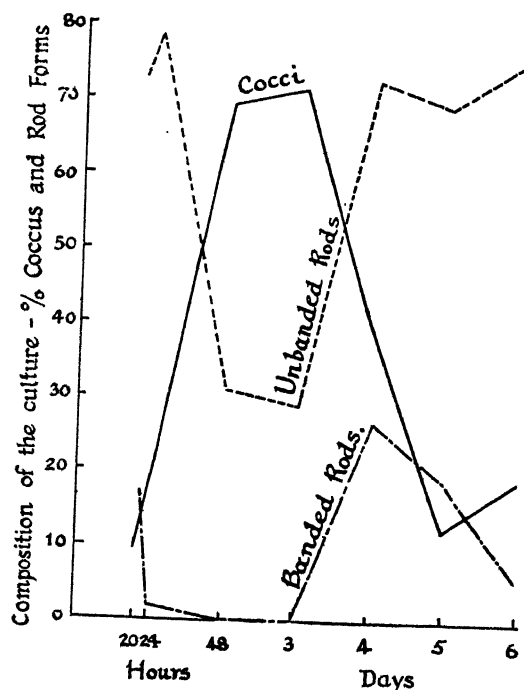


FIG. 5.—Changes in the life-cycle of *Bacillus radicicola* in soil inoculated with a suspension in water + calcium phosphate.

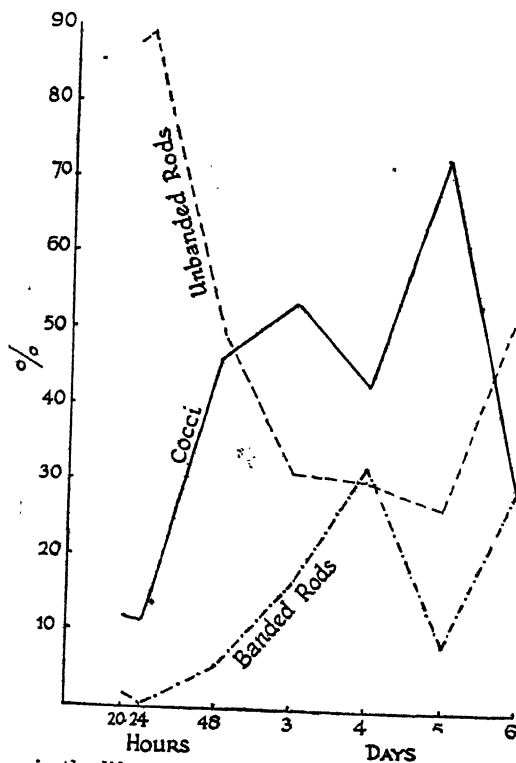


FIG. 6.—Changes in the life-cycle of *Bacillus radicicola* in soil inoculated with a suspension in milk.

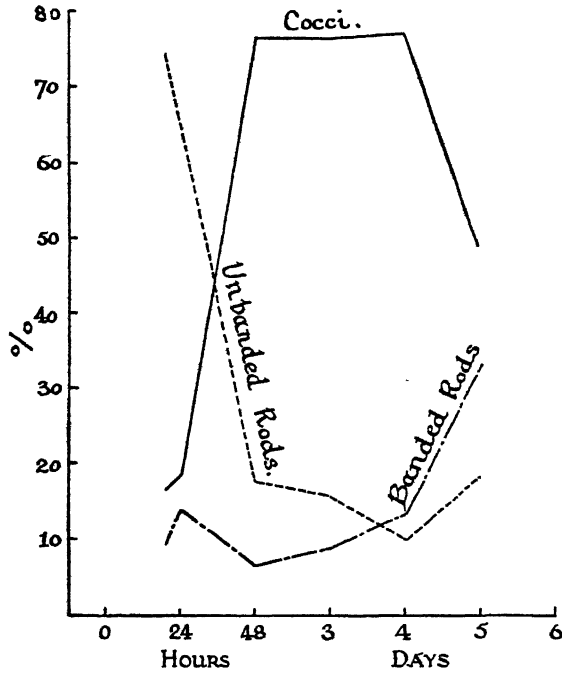


FIG. 7.—Changes in the life-cycle of *Bacillus radicicola* in soil inoculated with a suspension in milk + calcium phosphate.

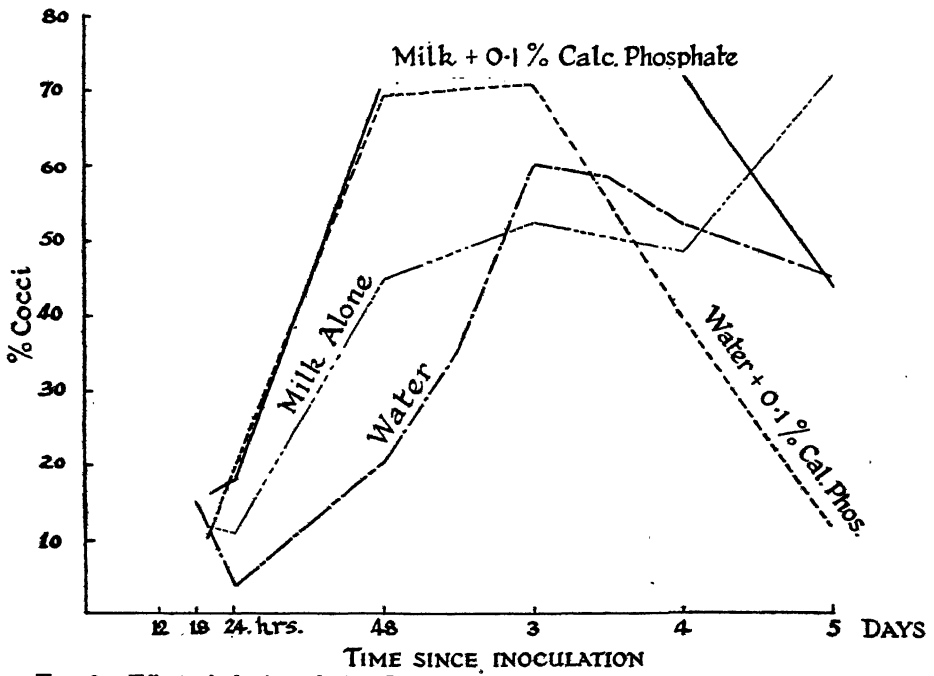


FIG. 8.—Effect of the inoculating fluid on the appearance of the coccus stage in soil.

off. The addition of calcium phosphate to the inoculating fluid, whether this be milk or water, causes a rapid preponderance of cocci, which, within 48 hours, form about 70 per cent. of the population. The use of a milk inoculum lengthens the time during which the cocci predominate.

Since a rise in the percentage of cocci coincides with the appearance of motile forms in the soil, the different inoculating fluids, which modify the time of coccus predominance, should also influence the time of appearance of the motile cells in the culture. To determine how soon motile forms appeared when the bacterial suspensions in the various fluids used above were inoculated into soil, suspensions of a 10-days' old agar culture of *Bacillus radicumicola* were made in these fluids. These suspensions were used to inoculate test-tubes containing 25 grams of sterile soil and sand mixture. Hourly examinations were made, fresh preparations being searched for motile organisms. The times when motile forms were first observed are recorded in Table II.

Table II.—Hour of Appearance of Motile Forms (from the Time of Inoculation).

Inoculum.

Tap water	Between 18th–20th hour.
Skim milk	Between 15th–18th hour.
0.1 per cent. calcium phosphate in water	Between 18th–20th hour.
Skim milk + 0.1 per cent. calcium phosphate.....	Between 10th–12th hour.

Cultures added to the soil in suspensions, either in water or in a solution of phosphate in water, take the same time to produce motile forms in the soil. A milk suspension, however, produces motile forms earlier, and the addition of the phosphate to the milk greatly shortens the time of their appearance as compared with milk alone.

E. SPREAD OF THE BACTERIA THROUGH STERILE SOIL.

If the bacteria move actively through the soil, the nature of the inoculating fluid, which alters the time of appearance of the motile coccus stage, should similarly affect the time of commencement of spread from the point of inoculation.

Previous workers differ as to the importance of active motility in influencing the spread of *Bacillus radicumicola* through soil. Ball (1909) (1) found that, in suitably moist soil, the bacteria diffused at a rate of 1 inch in 48 hours. He concluded that movement was largely due to soil-water currents aided by multiplication and motility of the organism. Kellerman and Faucett (9) found that in saturated soil, at 25° C., the bacteria moved at a rate of 1 inch in 48 hours. In barely moist soil, or at 10° C., this rate was reduced to about 1 inch in 72 hours. They assumed, however, that the movement was due to

the multiplication of the bacteria. Frazier and Fred (1922) (6), however, concluded as a result of pot and field experiments, that soy-bean nodule bacteria spread very slowly, if at all, unless passively carried by water currents.

In the present work, the spread from the centre of petri dishes of soil was studied as described in the footnote.* The bacteria spread evenly in each direction (fig. 9). Their migration was, therefore, little affected by inequalities of soil conditions in the petri dish.

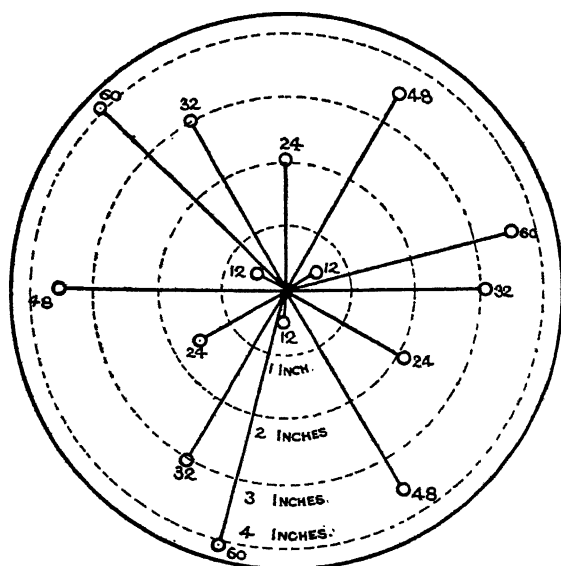


FIG. 9.—Radial spread of *Bacillus radicicola* from the centre of a petri dish of soil. The straight lines show the distances reached in each direction in the number of hours indicated.

* Petri dishes $8\frac{1}{2}$ in. in diameter were filled each with 400 grams of the soil and sand mixture as used in experiment 1, and the moisture content brought up to 18 per cent. The lid of the petri dish was lined with filter paper to prevent condensation water from dripping off the lid on to the soil. The petri dishes were sterilised in the autoclave. A cork-borerful of soil-sand mixture was removed from the centre of each petri dish, and the space thus left was filled with air-dry soil and sand, previously sterilised. Upon this was placed one drop of bacterial suspension made up in the inoculating fluid to be tested. The small column of air-dry soil-sand mixture absorbed the drop and prevented a local accumulation of moisture, which might have led to water currents carrying the bacteria outward. The dishes were incubated at 22°C ., and, at intervals, samples were taken with a sterile cork borer every $\frac{1}{2}$ in. along three radii. From each sample preparations were made, following the technique used for the life-cycle studies, and a loopful of each sample was streaked upon a slope of lucerne root-extract agar. The presence or absence of the organism at given distances from the centre along three different radii was then determined by examination of stained preparations, and the observation confirmed by the presence or absence of growth on agar slopes. There was a close agreement between the results of these two methods.

Fig. 10 shows the results of a number of trials in which bacterial suspensions in water alone, in milk alone, and in milk + 0.1 per cent. $\text{CaH}_2(\text{PO}_4)_2 + 2\text{H}_2\text{O}$ were used to inoculate the centre of the dish. The trials were made in duplicate, using water and milk suspensions, and in triplicate, using milk and phosphate, the curve on the left of the figure representing two trials, the results of which were identical. The nature of the inoculating fluid greatly influenced the time at which spreading commenced.

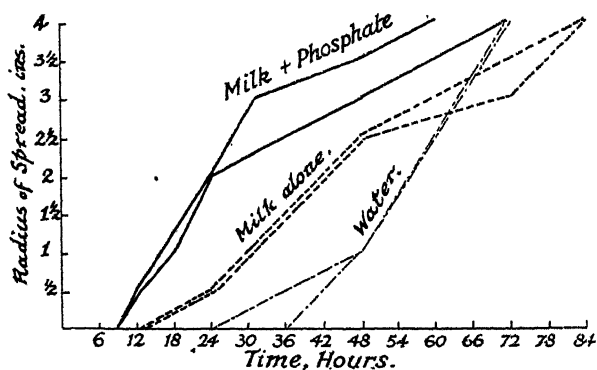


FIG. 10.—Influence of the inoculating fluid on the spread of *Bacillus radiculicola* through soil.

In fig. 11 the influence of the three inoculating fluids on the spreading is compared with their influence on the percentage of cocci found in soil at the same intervals of time during the life-cycle studies. The inoculating fluid similarly affects the time of coccus formation and the time at which spreading commences, suggesting that the motile cocci actively migrate through the soil.

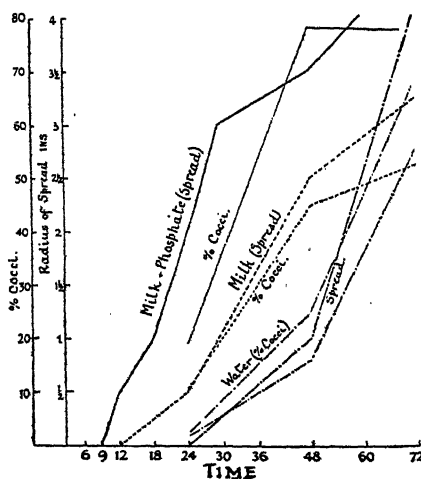


FIG. 11.—Influence of the inoculating fluid on the spread of *Bacillus radiculicola* and on the percentage of cocci formed in soil.

In this experiment, the organisms, in the course of spreading, must soon have removed themselves from the direct influence of the inoculating fluid, since the inoculum was added only to the centre of the dish. A more rapid spread, however, by distributing the population through a larger volume of soil, would tend to increase the available food supply and might thus exert a comparatively remote influence on the multiplication of the organisms. By the use of the technique described in the footnote,* the effect of the inoculating fluid on bacterial numbers was studied.

Fig. 12 shows the distribution of bacterial numbers in troughs of soil and sand mixture inoculated at one end with a bacterial suspension in water. After 24 hours the organisms are concentrated near the point of inoculation, the soil beyond the second inch being sterile. By the fourth day little increase in total bacterial numbers is found, but the bacteria are now dis-

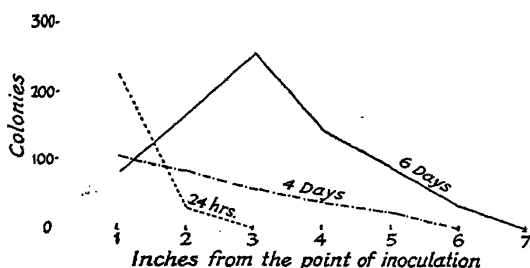


FIG. 12.—Numbers of *Bacillus radicicola* in soil at varying distances from the point of inoculation. Inoculum suspended in water.

* 1500 grams of soil-sand mixture were placed in a "Pyrex" glass trough 3 in. deep, measuring 10 in. by $4\frac{1}{4}$ in. at the top, and having a floor measuring 8 in. by 3 in. The moisture content of the mixture was made up to 18 to 19 per cent. A cork-borerful of soil was removed from the end of the trough and replaced by small beads. Care was taken to pack the beads closely so that they were in contact with the surrounding soil. A ridged zinc cover, having a hole at each end of the ridge, and with the sides projecting downwards 1 in. all round, was made to fit over the glass trough. The holes and the space between the projecting metal sides and the glass trough were packed with cotton-wool, and the apparatus was sterilised at 15 lb. pressure for half an hour.

The bacterial suspensions were prepared with the three inoculating fluids—(1) water alone, (2) skim milk alone, and (3) skim milk + 0.5 per cent. di-acid calcium phosphate. The glass beads were just moistened with the suspension to be tested, and the trough then incubated at 22° C.

For each inoculating fluid three troughs were used, and bacterial counts were made at intervals of one, four and six days, one trough being opened on each occasion. Duplicate samples were taken 1 in. away from the point of inoculation, and at intervals of 1 in. up to the eighth inch, and plate counts of the bacteria were made from each sample. A close agreement between the duplicate samples was always found.

tributed as far as the fifth inch. By the sixth day the organisms have reached the sixth inch, and a considerable increase in numbers has taken place. As compared with the numbers after four days, there has been an increase of about four-fold at the third, fourth and fifth inches, but at the second inch the numbers are less than doubled, while at the first inch there has been an actual decrease in numbers. This distribution of numbers indicates that the organisms are better able to multiply in the recently infected soil than near the point of inoculation, where their previous growth has rendered the soil a less favourable medium. Actual migration of motile forms may be a contributory cause.

Fig. 13 shows the distribution of numbers in troughs inoculated with a suspension in milk. Here, again, a multiplication near the point of inoculation occurs in the first 24 hours, and subsequently spreading occurs, the bacteria reaching the fifth inch on the fourth day and the sixth inch on the sixth day.

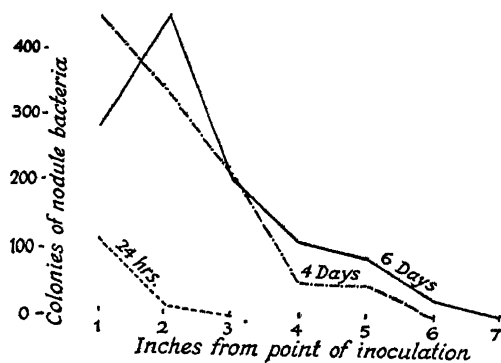


FIG. 13.—Numbers of *Bacillus radicola* in soil at varying distances from the point of inoculation. Inoculum suspended in milk.

The milk, however, produces a great effect in stimulating multiplication as compared with the water inoculum. This stimulation is, however, confined to the region neighbouring the point of inoculation, the numbers found beyond the second inch on the sixth day being no higher than was the case with the water inoculum.

Fig. 14 shows the distribution of numbers in troughs inoculated with a suspension in milk + 0.5 per cent. $\text{CaH}_2 + (\text{PO}_4)_2 \cdot 2\text{H}_2\text{O}$. The addition of the phosphate causes a marked increase in multiplication, and also in spreading, the bacteria reaching the farther end of the trough within four days. There is a considerable fall in numbers at the first and second inch on the fourth and sixth days, and this is perhaps due to the high numbers in that region after 24 hours.

The addition of the phosphate, however, produces a very great increase in numbers from the third to the fifth inch. By the sixth day the region of highest numbers has moved forward to the fourth inch, where the numbers are more than three times as great as with the milk inoculum without phosphate. In considering what is the cause of this increase, the small amount of phosphate added to the trough must be recognised. About 0.25 c.c. of the bacterial suspension in milk + calcium phosphate was used to inoculate the end of each trough. The 1.25 mgs. of phosphate contained in this can scarcely have exerted an appreciable direct action on the number of organisms four and five inches from the point of inoculation. The curves, however, show that the bacteria increase most rapidly in the newly infected soil, so that the known effect of the phosphate

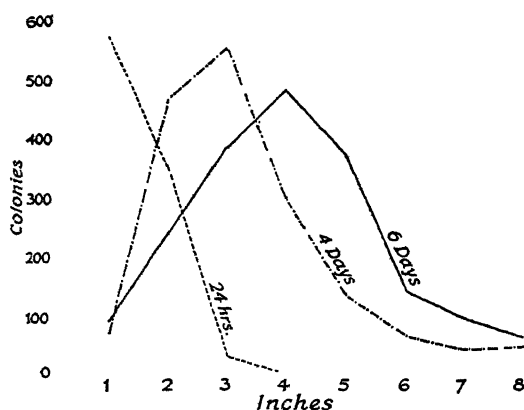


FIG. 14.—Numbers of *Bacillus radicicola* in soil at varying distances from the point of inoculation. Inoculum suspended in milk + calcium phosphate.

in hastening migration of the bacteria through the soil supplies an explanation of its remote influence on the multiplication. The invasion of fresh soil by motile individuals is followed by a wave of high bacterial numbers, due to multiplication of the bacteria in the freshly invaded soil. The action of the phosphate in stimulating the spreading consequently results in an extension of the wave of high numbers to a greater distance from the inoculation point in a given time. The addition of calcium phosphate to the milk used as inoculating fluid thus produces a two-fold effect on the subsequent distribution of the bacteria in the surrounding soil:—first, it enlarges the volume of soil infected in a given time, and, secondly, it increases the number of organisms within that volume. In the practice of seed inoculation, both these factors should increase the chances of root-infection, and consequently favour the formation of nodules.

F. INFLUENCE OF THE INOCULATING FLUID ON NODULE FORMATION.

A series of pot experiments were made to see whether the addition of calcium phosphate to the inoculating fluid would increase the formation of nodules.

Pot Experiment 1.

Thirty cylindrical glazed earthenware pots, $6\frac{1}{2}$ inches in diameter and 15 inches deep, were each filled with 22.5 lb. of sieved garden soil, to which 30 per cent. sand had been added. The moisture content of this mixture was made up to about 20 per cent. before filling the pots. The soil used was one in which lucerne nodule bacteria were present in small numbers, and in order to test the additional effect of the inoculation in such soil, neither soil nor seed were sterilised before use. Thick suspensions of the bacteria were made in skim milk alone, and in skim milk to which 0.1 per cent. di-acid calcium phosphate had been added. Uniform weights of seed were wetted with equal volumes of each suspension and the seed sown at the rate of 1 gram per pot. Ten parallel pots with each type of inoculation were set up and ten pots were sown with uninoculated seed. All pots received equal watering and the plants were thinned out in successive stages, so that at the sixteenth week, five plants per pot remained. Nodule counts were made after 4, 5, 6, and 16 weeks. For this purpose the roots were washed with a fine jet of water from a hose. In counting the nodules, the roots were floated in a shallow dish and examined with a lens against a black surface. Dry weights of crop were also taken.

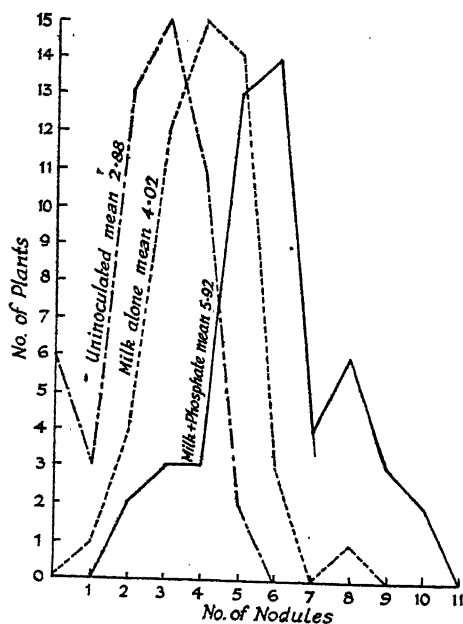


FIG. 15.

The distribution of nodules on individual plants at the fourth, fifth, and sixteenth weeks is shown in the form of frequency curves in figs. 15, 16, and 17. The effect of the phosphate is increasingly evident with the age of the plant,

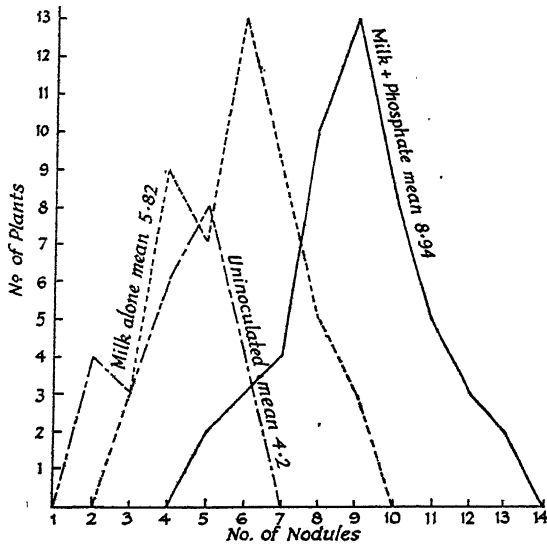


FIG. 16.

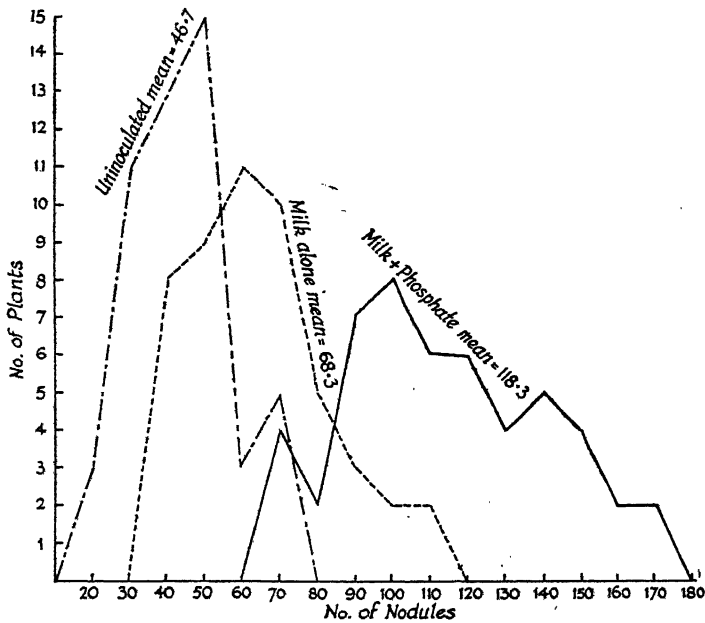


FIG. 17.

but it is undoubtedly significant even at the fourth week. There has also been an effect on the weight of the plants, as is seen in Table IV.

Table IV.—Showing the Yield of Lucerne at the Sixteenth Week.

Treatment.	Mean number of nodules.	Mean dry weight.		Nodules per gram of roots.
		Tops.	Roots.	
Uninoculated	47·3	9·05	12·67	3·8
Milk inoculum	65·2	10·72	13·66	4·7
Milk 0·1 per cent. calcium phosphate inoculum	116·2	12·71	14·42	8·07

Pot Experiment II.

In this experiment the soil and sand mixture was steamed for eight hours, and sterilised skim milk was used in making up the inoculating suspensions, but the seed was not sterilised. An additional series of pots was set up, sown with seed inoculated with a suspension of bacteria in 0·1 per cent. solution of di-acid calcium phosphate in distilled water. Eight parallel pots of each treatment and six pots of uninoculated seed were sown, each pot receiving 2 grams of seed and the seedlings being thinned out as before. Nodules were counted on the second, third, fourth, fifth and sixteenth weeks, and the results are shown in Table V.

Table V.—Showing the Mean Number of Nodules per Plant.

Age of plant.	No. of plants.	Uninoculated.	0·1 per cent. phosphate inoculum.	Milk inoculum.	Milk and 0·1 per cent. phosphate inoculum.
2nd week	50	2·04	3·00	3·38	3·28
3rd week	50	2·42	3·20	3·70	3·67
4th week	50	1·90	3·86	3·78	4·18
7th week	20	10·65	19·6	15·45	15·9
16th week	10	43·8	56·2	59·0	113·1

The addition of phosphate to the milk has again caused a big increase on nodule numbers by the sixteenth week (see fig. 18), though there is no significant effect before this.

The plants were harvested and dry weights taken at the sixteenth week, but five pots of each treated series and three of the uninoculated were left after cutting the crop, and the second growth after 30 weeks was cut and weighed. The effect of the milk phosphate on the growth is more marked at the thirtieth week than after 16 weeks (Table VI).

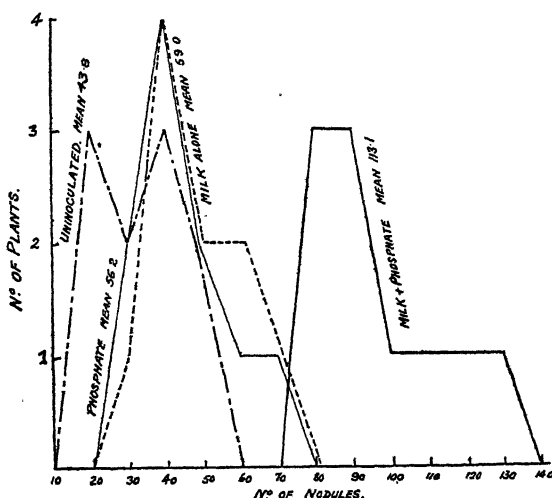


FIG. 18.

Table VI.—Pot Culture Trial of Lucerne Inoculation.

Treatment.	Result after 16 weeks.		Result after 30 weeks.	
	Mean No. of nodules.	Dry weight of tops.	Dry weight of tops.	Dry weight of roots.
Uninoculated	43.8	12.6	9.2	11.5
Inoc. milk suspension	59.0	12.1	9.8	12.1
Inoc. suspension in aqueous salt calc. phos. 0.1 per cent.	56.2	13.9	9.9	12.8
Inoc. suspension in milk 0.1 per cent. calc. phos.	113.1	14.4	12.6	15.5

Pot Experiment III.

In this experiment, 25 pots were used, each containing 22.5 lb. of soil-sand mixture.

bacteria at the rate of 13,600 bacteria per seed. The pots were watered at intervals with boiled water. Fig. 19 shows the numbers of nodules which developed after five weeks.

This experiment confirms the effect of adding di-acid calcium phosphate to milk in increasing nodule development. The addition of the phosphate, however, has in no way improved tap water as an inoculating fluid.

G. DISCUSSION.

The pot trials above described show the increased nodule formation from seed inoculated with a bacterial suspension in milk + di-acid calcium phosphate over that inoculated with a suspension in milk alone. The dose of phosphate that produces this effect is minute. Approximately 0.2 c.c. of the bacterial suspension was used to wet 1 gram of seed, and this volume contained 0.1 per cent. of calcium phosphate. The amount of calcium phosphate added to each pot containing about 20 lb. of soil was therefore about 0.2 mg. in pot experiments I and III and 0.4 mg. in experiment II. So small a dose can scarcely have had any direct influence on the plant growth. It has been shown, however, that doses of this order or magnitude, by affecting the production of motile forms of the bacteria, do influence their migration through the soil and exert a remote influence on the bacterial numbers. The increased nodule formation can therefore be attributed to the wider distribution of the bacteria and to their greater numbers at a distance from the seed.

If this explanation is correct, the added calcium phosphate should show its effect in increasing the nodule numbers towards the extremities of the roots rather than near the seed. In order to test this point the following experiment was carried out:—32 glazed earthenware pots were filled with soil and sand mixture. Half of them were sown with lucerne seed inoculated with a bacterial suspension in milk + 0.1 per cent. $\text{CaH}_4(\text{PO}_4)_2 + 2\text{H}_2\text{O}$ and half with seed inoculated with a suspension in milk alone. A standardised inoculum was used, so that each seed received approximately 4,500 bacteria. 0.25 gram of seed was sown per pot and the plants thinned out as seemed necessary. At intervals during the growth period duplicate pots of each type were removed, the roots washed and separate counts of the nodules in the top 4 inches of the root and below that depth were made. The development of nodules in the surface region and in the deep region is shown in fig. 20.

The addition of calcium phosphate produces no increase in nodules in the top four inches of the root, near the point of inoculation. There is consequently

no effect from the phosphate until the sixth week, when the root system begins to develop below this depth. From this time onwards the phosphate produces an increase in nodule numbers, which, however, is entirely confined to the distal region of the root system. The effect of the phosphate consequently becomes more marked as the plant grows older and the lower roots develop.*

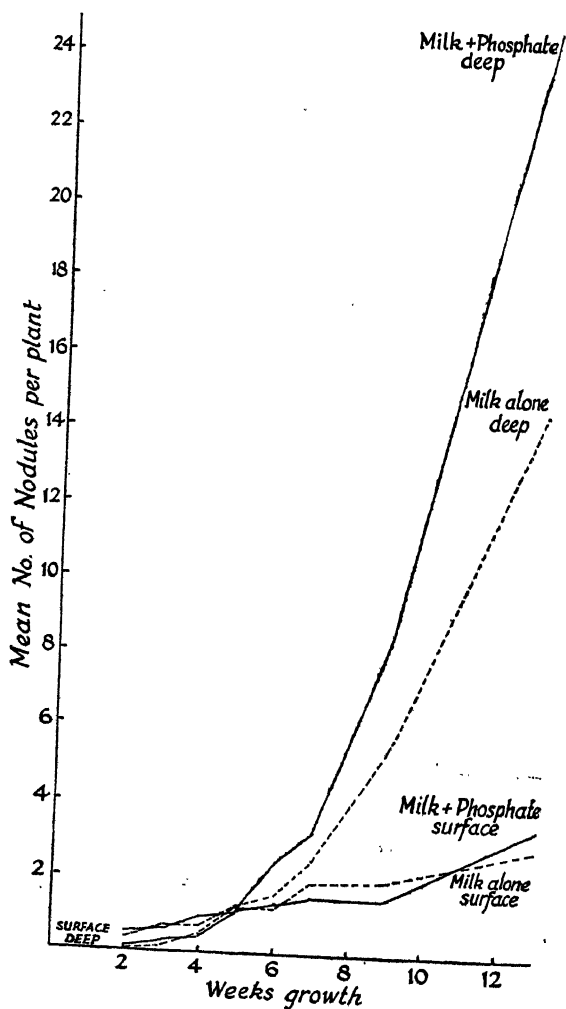


FIG. 20.—Influence of the inoculating fluid on the number of nodules produced near the surface and in the deeper parts of the root system.

This increasing effect of the phosphate with the age of the plant is also seen in pot experiments I and II (fig. 21).

* The authors are gratefully indebted to Mr. P. H. H. Gray for his assistance in making counts of the nodules in this and in the other pot experiments.

While the effects produced by phosphate are thus in accordance with the above explanation, they also show that the spread of the organisms in the soil of the pots cannot proceed and continue at the rate recorded in the laboratory experiments. The rate of radial migration in petri dishes was found to be of the order of one inch in 24 hours, while in the glass-trough experiments the wave of high bacterial numbers appears to move along the trough at an approximate rate of one inch in 48 hours. While the experimental data on this point are still insufficient, they indicate a rate of migration which, if maintained in the pot trials, would result in the infection of the whole mass of soil in about 30 days, even with the least efficient method of inoculation. Measurements of spreading have not, however, been carried out for long enough period to decide to what

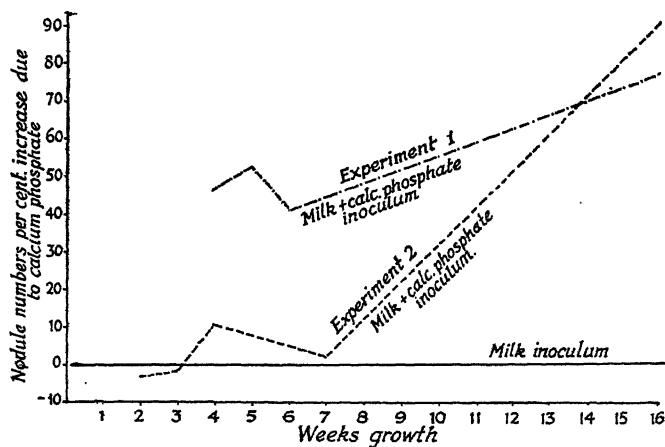


FIG. 21.

extent it is maintained, and the pot experiments suggest either that the initial rate of spread is not maintained or else that, under the different soil conditions in the pot, it occurs at a sufficiently slow rate to enable the addition of phosphate to produce an effective increase in bacterial numbers in the lower layers of soil even later than six weeks after sowing.

The effect of calcium phosphate, as shown in the above experiments, has a bearing on the commercial methods of seed inoculation. The idea of inoculating seed with pure cultures of the nodule bacteria was first conceived by Nobbe (13) in 1896 in collaboration with Hiltner. The method of seed inoculation has since been tested extensively with varying success in many parts of the world, and in these trials a number of different liquids have been used in making the bacterial suspension with which the seed was wetted. In 1902 the use of a suspension of the bacteria in skim milk as the inoculum was tried, and this method is now

used extensively and with success in Denmark for the inoculation of lucerne seed. The addition of di-acid calcium phosphate to the milk is thus a simple modification of an already existing practice, and the cost of it should be negligible on account of the very small doses required.

H. SUMMARY.

1. By means of a modification of Winogradsky's staining technique the changes in morphology of *Bacillus radicicola* in soil were followed. A regular cycle of changes was found, unbanded rods, cocci, and banded rods successively predominating in the soil. Increase in the percentage of cocci was associated with increased bacterial numbers and with the appearance of motile forms (Section C).

2. By modifying the liquid used to suspend the inoculum added to the soil, the time of appearance of cocci in predominance could be altered. In particular, inoculation of the soil with a bacterial suspension in milk + 0.1 per cent. $\text{CaH}_4(\text{PO}_4)_2 + 2\text{H}_2\text{O}$ hastened the predominance of cocci and increased the percentage to which they attained (Section D).

3. When the centre of a petri dish of soil and sand is inoculated with a suspension of the bacteria, the latter commence, after a lag period, to spread radially at an approximate rate of one inch in 24 hours. The length of this lag period is apparently related to the time taken for cocci to predominate in the soil and is similarly effected by the nature of the inoculating fluid. The bacteria multiply rapidly in the soil into which they have recently spread, so that the nature of the inoculating fluid also exerts a remote influence on bacterial numbers. Thus inoculation of the soil with a bacterial suspension in milk containing 0.1 per cent. $\text{CaH}_4(\text{PO}_4)_2 + 2\text{H}_2\text{O}$ results in a greater spreading of the bacteria through the soil and in greater multiplication at a distance from the point of inoculation than in the case when soil is inoculated with a suspension in milk alone (Section E).

4. Lucerne plants grown from seed inoculated with a suspension of bacteria in milk + 0.1 per cent. $\text{CaH}_4(\text{PO}_4)_2 + 2\text{H}_2\text{O}$ showed a considerable increase in nodule numbers and in yield compared with plants from seed inoculated with a suspension in milk alone (Section F).

5. This effect was confined to the deeper portions of the root and therefore increased as the plants became older and roots developed in the deeper soil. This suggests that the additional nodule formation is due to the known effect of the phosphate in increasing the spreading of the bacteria (Section G).

REFERENCES.

- (1) Ball, O. M., 'Centralbl. f. Bakt.,' Abt. II, vol. 23, p. 47 (1909).
- (2) Beijerinck, M. W., 'Botan. Zeit.,' vol. 46, pp. 726, 758, 782, 798 (1888).
- (3) Bewley, W. F., and Hutchinson, H. B., 'Journ. Agric. Sci.,' vol. 10, p. 144 (1920).
- (4) Brunchorst, J., 'Berichte Deut. Botan. Gesell.,' vol. 3, p. 241 (1885).
- (5) Buchanan, R. E., 'Centralbl. f. Bakt.,' Abt. II, vol. 23, p. 59 (1909).
- (6) Frazier, W. C., and Fred, E. B., 'Soil Sci.,' vol. 14, p. 29 (1922).
- (7) Gangulee, N., 'Annals of Applied Biol.' (*in the press*).
- (8) Greig-Smith, R., 'Proc. Linn. Soc. N.S. Wales,' vol. 24, p. 653 (1900).
- (9) Kellerman, K. F., and Faucett, E. H., 'Science, N.S.,' vol. 25, p. 806.
- (10) Löhnis, F., 'Mem. Nat. Acad. Sci.,' Washington, vol. 16 (1921).
- (11) Löhnis, F., and Smith, N. R., 'Journ. Agric. Res.,' vol. 6, p. 675 (1916).
- (12) Morck, D., 'Diss. Phil.,' Leipzig, 44 pp. (1891).
- (13) Nobbe, F., 'Botan. Centralbl.,' vol. 68, p. 171 (1896).
- (14) Prazmowski, A., 'Landw. Vers. Stat.,' vol. 37, p. 161 (1890).
- (15) Wallin, I. E., 'Journ. Bact.,' vol. 7, p. 471 (1922).
- (16) Winogradsky, M. S., 'Chimie et Industrie,' vol. 2, No. 2 (1924).

*Ultra-Violet Radiation and Metabolism, with a New Method
for Estimating Metabolism.*

By J. ARGYLL CAMPBELL.

(Communicated by Dr. Leonard Hill, F.R.S.—Received January 20, 1926.)

(From the National Institute for Medical Research, Hampstead.)

[PLATE 17.]

Introduction.

Kestner (1) and his co-workers recently found that ultra-violet radiation produced an immediate, but temporary, rise in metabolism of adults and children. Fries (2) employed healthy children and could find no material effect upon metabolism as the result of such radiation, and states that the rise of metabolism which may possibly occur in certain children is not due to radiation. It must be remembered that during a light bath the skin of a human being is not only exposed to rays but also to the air.

Krogh (3) reviewing the literature concludes that light has no effect, if movement of the subjects is properly controlled. Neither Hasselbalch nor Durig, whose work has been reviewed recently by Hausmann (4), observed any effect upon metabolism as the result of radiation.

Leonard Hill and myself (5) carried out a series of observations upon human subjects undergoing heliotherapy and open-air treatment, at Alton in England, and at Montana in Switzerland. We were not able to attribute any of the increase in metabolism we observed to radiation from the sun. We followed their metabolism for several months and found the metabolism much higher in winter with little sun, than in summer with maximum sun.

We also carried out observations upon six subjects before and after exposure to light baths over a period of $5\frac{1}{2}$ months, using the carbon-arc lamp (amps. 20 volts 60) for treatment of lupus. This research was carried out in a ward at the London Hospital with the co-operation of Dr. Sequeira. In four of the subjects we observed no effect upon metabolism following prolonged radiation from the carbon arc lamp; in the other two subjects there was a definite rise, the interpretation of which was difficult, owing to the approach of puberty. There was a distinct improvement in the lupus in all of them. In this research the air surrounding the subjects was comfortably warm.

More recently Harris (6) has published results claiming that, although the total rays from either the iron-arc lamp or mercury-vapour lamp have no effect upon metabolism, yet there is a group of rays at the violet end of the spectrum, namely those with wave-lengths from 436–291 $\mu\mu$, which have a marked and immediate effect upon the metabolism of mice and rats, an increase of 20 per cent. in the output of CO_2 being observed. He obtained this group of rays by interposing a screen of blue uviol glass between the animal and the source of light.

Experiments similar to those of Harris have been performed, using the mercury vapour lamp, and the results, which do not support his claim, are given in the present paper.

Methods.

White mice, white rats and human subjects were employed. The metabolism of the human subjects was estimated by the Douglas-Haldane method, as recommended by Cathcart (7).

The metabolism of mice and rats was estimated by two methods; one of them was similar to that used by Harris; in this the CO_2 output was estimated by the Haldane-Pembrey method in which the CO_2 is absorbed by soda-lime and weighed. It was not convenient to estimate the O_2 consumption by this method, which requires weighing of the animal chamber, because, in order to keep this chamber at constant temperature during radiation, it was sometimes

necessary to apply water to the outside of the chamber. The drying of the chamber required considerable time and was not a simple matter. It was necessary therefore to find some other method to estimate the O_2 intake of mice and rats.

After several unsuccessful attempts with face masks a circulation method which seemed to be reasonably accurate, was devised. A diagram of the apparatus is shown in fig. 1. A definite volume of air was placed in a Douglas

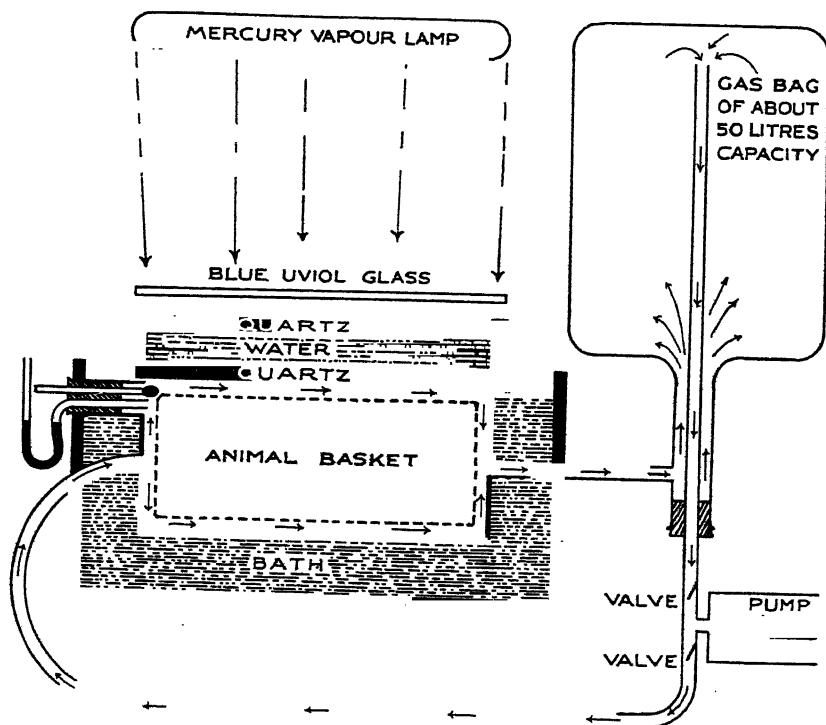


FIG. 1.

bag, and by a suitable pump this air was pumped from the bag through the animal chamber back to the bag again. In this way the air was kept in continuous circulation for an hour. To ensure proper mixing of the air in the bag the inlet to the bag was placed at a distance from the outlet; the outlet was a long rubber tube with its end near the bottom of the bag; the inlet was at the top of the bag.

The contents of the bag were analysed for CO_2 and O_2 both before and after one hour's circulation through the chamber. The volume of air in the bag was measured before and after the circulation. The animal produced CO_2 and

consumed O_2 , so that there was an increase of CO_2 and a decrease of O_2 in the gas in the bag. The volume of air placed in the bag at the start was such that the CO_2 produced in one hour would not form more than 1 per cent. of the total volume in the bag; in the same time the O_2 fell about 1.3 to 1.4 per cent., that is from 20.93 to 19.63 or 19.53. For a rat about 120 grammes about 20 litres was found to give the required condition since a rat of this size produces about 3.5 c.c. CO_2 per minute. This means that at the start the animal is breathing pure air, but in half an hour the CO_2 in the circulating air forms about 0.5 per cent.; in three-quarters of an hour about 0.75 per cent., and in one hour about 1.0 per cent. The breathing of such a quantity of CO_2 as 1 per cent. in the air does not affect the CO_2 discharge to any significant extent, as pointed out by Haldane and others (8). In any case, 0.5 per cent. CO_2 has been detected in the vicinity of rats and mice lying in their glass jars. They lie, with their heads underneath, in heaps in a nest in a corner, and the animals at the bottom of the heap must be accustomed to such a quantity of CO_2 . Moreover, the results for CO_2 output with the circulation method were similar to those obtained with the Haldane-Pembrey method.

Table I gives results of experiments lasting 5 hours, the CO_2 being estimated over three separate hourly periods in this time. It will be observed that the general agreement of the methods was close; the experiments compared were performed on different days, using the same animals; in the case of the rats, the weights had changed somewhat.

Table I.—Comparison of Methods.

Animal.	CO_2 cubic centimetre per minute.					
	Haldane-Pembrey.			Circulation.		
	1st	2nd	3rd	1st	2nd	3rd
Rat 100- ⁹⁵ G	2.95	2.88	3.58	2.89	2.69	2.93
„ 140- ¹²⁰ G	3.80	3.73	4.03	3.99	3.25	3.51
6 Mice 120 G	3.29	3.26	3.24	3.59	3.45	3.29

In the third hourly period with the Haldane-Pembrey method, in the case of the rat weighing 100 gm., there appears to be a marked increase in CO_2 output. Such an alteration was observed occasionally (1 in 8 experiments) with either method. It did not appear to be due to movement; it was possibly due, in part, to alteration in tone of the muscles, or to thyroid activity.

Comparison of the results given in Tables II and III also reveals the agreement of the results obtained by the two methods.

In most of the experiments, the CO₂ output fell very gradually from hour to hour, but only by a few per cent.

The advantage of the circulation method was that the O₂ consumption was also readily obtained. It was possible to analyse CO₂ and O₂ within an error of ± 1 per cent. The volume of air was measured within an error of ± 1 per cent. when the volume was 20 litres. A dry and a wet meter were used, the former being slightly more accurate.

The whole apparatus was carefully tested to determine whether any CO₂ or O₂ would disappear in a blank experiment due to absorption by the material of the apparatus; in the routine followed no such absorption occurred. Before the 20 litres of air was placed in the bag for each experiment, the bag had contained, and been flushed out with, air containing 1 per cent. CO₂ and about 19.55 per cent. O₂; the same bag was used throughout.

Care was taken to test the whole apparatus for leaks, both by means of a manometer and by placing the chamber, bag and tubing under water. The volume of air in the bag was measured both before and at the end of an experiment, a very small difference being observed; this afforded further evidence that the apparatus was airtight. The total error of the circulation method was considered to be ± 2 per cent.

The space in the chamber was almost completely filled, the animals being placed sometimes in a wire enclosure with just sufficient space to turn round. The animals lived for three months in the room in which the experiments were performed. The temperature of this room was kept about 20° C. The temperature in the chamber was kept constant by means of suitable screens and water at room temperature: circulation of water was not required. In most of the experiments, the temperature in the chamber was about 22° C., the majority of the experiments being with rats and groups of mice. The animals were accustomed to the noises of electric motors, &c., and to the switching on and off of lamps; and also to the blue colour from blue glass, so that the blue colour of the blue uviol glass would not disturb them. They were also placed in the chamber on several occasions before the actual experiments were performed. In this way, when the experiment was performed, they were quite used to the conditions and settled down readily; as a rule, they appeared to sleep. They were watched for movement, and when movement was of any degree the results were discarded.

The experiments were commenced at 9.30 a.m., and lasted for about five

hours. The animals were fed about 11 a.m., except those used that day in the experiment; these were fed after the experiment, *i.e.*, at 2.30 p.m. All the animals seemed to take food before 5 p.m. In some cases, they fasted for 17 hours before the experiment. The results were much the same as when their food was left in their jars, probably because they did not feed at night in any case.

Results of Experiments with White Mice and Rats using the Haldane-Pembrey method.

The animals were exposed to the total rays of the mercury-vapour lamp (Cooper-Hewitt type, 3.5 amps. 110 volts between the poles; Kw. = 0.38) at 25 cm. distance, for $1\frac{1}{2}$ hours; then to the rays of this lamp through the blue uviol glass for $1\frac{1}{2}$ hours; and during the last period of $1\frac{1}{2}$ hours all the rays were shut off by an opaque screen. The spectra from the naked mercury-vapour lamp (223–770 $\mu\mu$) and from the blue uviol glass (291–436 $\mu\mu$) are compared in fig. 2 (Plate 17).

Not only does the blue uviol glass cut down the limits of the spectrum very greatly, but also it cuts down the intensity. Leonard Hill has found that when the naked mercury-vapour lamp kills infusoria in 5 minutes, the blue uviol rays kill in 40 minutes. The blue uviol glass was 0.75 mm. in thickness. The spectra and the relative time exposures give some indication of the density of the blue uviol (see fig. 2, Plate 17).

The mercury-vapour lamp was kept burning, but screened by opaque material over the third period of the experiment; in this way it was found easier to maintain a constant temperature in the chamber, during this period of "no radiation."

The period of exposure to the blue uviol rays was placed between the other two periods, which thus acted as controls. The CO₂ was collected for the last 60 minutes of each period.

In some experiments groups of mice were used; in others single mice and rats were employed. The results indicated that there was no effect upon CO₂ output by radiation with rays from blue uviol glass. There was direct evidence that the rays had affected the animals in other ways. The ears exhibited erythema a few hours after the exposures, and later in some cases destruction of ear tissue developed, owing to damage to tissue by the ultra-violet rays.

Four experiments were performed in which the visible rays alone from the mercury-vapour lamp were used. The technique and general plan was the same as for the experiments in Table II, but a glass screen was also interposed,

Table II.—Haldane-Pembrey Method.

Animals.	CO ₂ cubic centimetre per minute.				
	Weight. gramme.	Temperature of chamber.	Mercury- vapour lamp.	+ Blue uviol.	No radiation.
		°C.			
Mouse	40	22.0	1.44	1.46	1.57
„	28	22.0	1.23	1.34	1.33
„	24	20.0	1.23	1.15	1.08
„	20	24.7	0.77	0.68	0.75
„	19	23.0	0.71	0.68	0.72
„	19	23.0	0.92	1.00	0.92
Average.....	25	22.4	1.05	1.05	1.06
6 mice.....	120	23.0	3.29	3.26	3.24
5 „	100	23.5	3.20	3.09	3.01
6 „	100	23.5	3.47	3.57	3.25
Average.....	107	23.3	3.32	3.31	3.17
Rat	135	23.5	3.71	3.88	4.21
„	140	23.5	3.80	3.73	4.03
Average.....	137	23.5	3.75	3.80	4.12

which shut out most of the ultra-violet rays; thus the animals were exposed to visible rays of the colours used previously, but without the biologically active ultra-violet rays. No difference was observed between these results and those in Table II. There was, therefore, no indication that either visible rays of the colours used, or ultra-violet rays of any wave-length, affected the CO₂ output of healthy mice and rats as measured by the Haldane-Pembrey method.

Results of Experiments with Rats and Mice, using the New Circulation Method.

Single rats (100–150 gm.) and groups of 5 or 6 mice were employed. Table III, A, B, and C, give details of the results. In Table III, A, 1½ hours' exposure to the blue uviol rays followed 1½ hours' exposure to the total rays of the mercury vapour lamp. In Table III, B, the metabolism without any radiation preceded the exposure to rays from these sources. In Table III, C, the exposure to the blue uviol rays was placed between a period of "no radiation" and a period of exposure to the naked mercury-vapour lamp. By thus changing the order

of the exposure to the blue uviol rays it was considered that perfect controls for the experiment would be obtained. The details in the Tables III, A, B, and C, reveal no effect whatever upon CO_2 output or O_2 intake by radiation through blue uviol glass. Indeed, the figures in these tables would do very well for animals without any radiation at all.

Table III.=Circulation Method.

A

Animals.	Weight, grammes.	Tempera- ture of chamber.	Cubic centimetre per minute.			
			Mercury-vapour lamp.		+ Blue uviol.	
			CO_2 .	O_2 .	CO_2 .	O_2 .
Rat.....	135	°C. 22.0	3.48	4.52	3.52	4.56
"	135	21.5	3.41	4.11	3.58	4.68
"	143	23.5	4.34	4.79	4.40	4.65
"	130	23.5	3.73	4.62	3.61	4.37
Average.....	136	22.6	3.74	4.51	3.77	4.56
			(R.Q. 829)		(R.Q. 827)	

B

Animals.	Weight, grammes.	Tempera- ture of chamber.	Cubic centimetre per minute.					
			No radiation.		Mercury-vapour lamp.		+ Blue uviol.	
			CO_2 .	O_2 .	CO_2 .	O_2 .	CO_2 .	O_2 .
Rat	95	°C. 21.5	2.89	3.80	2.69	3.73	2.93	3.60
"	150	21.0	3.84	4.98	3.84	4.81	3.82	5.10
"	130	22.5	3.99	5.09	3.25	4.32	3.51	4.57
6 mice	120	21.0	4.12	5.01	3.75	4.47	3.50	4.47
6 "	100	21.0	4.16	5.62	4.03	4.94	3.43	4.46
Average	119	21.4	3.80	4.90	3.51	4.45	3.44	4.44
			(R.Q. 775)		(R.Q. 738)		(R.Q. 775)	

Table III—(continued).

C

Animals.	Weight, grammes.	Tempera- ture of chamber.	Cubic centimetre per minute.					
			No radiation.		+ Blue uviol.		Mercury-vapour lamp.	
			CO ₂ .	O ₂ .	CO ₂ .	O ₂ .	CO ₂ .	O ₂ .
Rat....	100	°C. 23.0	3.18	4.25	3.18	4.21	3.18	4.39
6 mice	100	21.5	3.43	4.52	3.38	4.65	3.30	4.55
Average	100	22.2	3.30	4.38	3.28	4.43	3.24	4.47
			(R.Q. 753)		(R.Q. 740)		(R.Q. 724)	

The figures obtained, with both methods used, for metabolism of rats and mice are similar to those given by other observers (9) if allowance be made for differences in conditions, *e.g.*, weight of animals, temperature, etc.

In some of the experiments the metabolism of mice lying in groups was obtained; in others the same mice were employed, but singly. It was observed that with the mice lying in groups the CO₂ output was lower per mouse than when each mouse was taken singly. Thus in 9 observations with 3 groups of mice, the average CO₂ output per mouse (19 gm.) per minute was 0.57 c.c.; the limits being 0.54 to 0.64; whilst in 9 observations with the same mice singly the average output was 0.79 c.c., the limits being 0.68 to 1.00. It would seem probable that when mice are lying in a heap their surface exposed is reduced and thus less heat is lost and less CO₂ is produced, thus confirming the law of surface area.

Results of Experiments with Human Beings.

In these observations the results of exposure of the skin, of the ventral or dorsal aspect of the trunk of adult men, to the rays from the mercury-vapour lamp through the blue uviol glass were compared either with the results of visible rays alone or with the figures for normal resting metabolism. The subject sat comfortably on a chair in a dark room and the skin was radiated through a window of a well-ventilated chamber enclosing the mercury-vapour lamp. The window had an area of 274 sq. cm., this also being the area of the blue uviol screen used, so that up to 5 per cent. of body surface could be

radiated, the subject sitting 30 to 50 cm. from the lamp. The lamp was placed about 20 cm. from the window. The general conditions were kept constant; and body temperature, temperature of radiated skin and pulse rate were noted. All degrees of erythema, from slight to very marked with blistering, resulted as the after-effect of the exposures. Each part of an experiment covered at least one hour, so that the skin was exposed for this time to the rays through the blue uviol glass. The metabolism was estimated over the last 15 minutes of each period.

The results are given in Table IV, and reveal no effect, either immediate or delayed, upon metabolism by radiation through blue uviol glass; nor did radiation with visible rays alone produce any significant change from normal.

Table IV.—Results with Human Beings.

Subject.	Surface radiated.	O ₂ consumption increase (+) or decrease (—) by blue uviol.	Erythema.
C.P.	674 sq. cm. ventral	— 9.0	v. marked.
	639 " " dorsal	+ 0.9	fair.
	929 " " "	— 5.0	marked.
	735 " " ventral	— 5.2	marked.
Y.A.	773 " " "	± 0.0	fair.
	923 " " dorsal	+ 2.6	marked.
L.H.	773 " " ventral	+ 4.0	marked.
	697 " " dorsal	— 5.5	fair.
J.A.C.	773 " " ventral	— 1.2	marked.

Summary and Conclusion.

(1) Radiation by total rays (223–770 $\mu\mu$) from the mercury-vapour lamp, or by the group of rays through blue uviol glass (290–436 $\mu\mu$), or by the visible rays from either of these sources (400–770 $\mu\mu$ and 400–436 $\mu\mu$ respectively) has no effect upon the metabolism of healthy men, mice or rats.

(2) When mice lie together in groups there is apparently a decrease of CO₂ output due to reduction of surface area.

(3) A new method for estimation of CO₂ output and O₂ intake is described.

The subject of this research was suggested by Dr. Leonard Hill, F.R.S., to whom I am indebted for kindly criticism and advice. My thanks are also due to Dr. Purdy for the diagram and to Mr. J. E. Barnard, F.R.S., whose department supplied the photograph of the spectra.

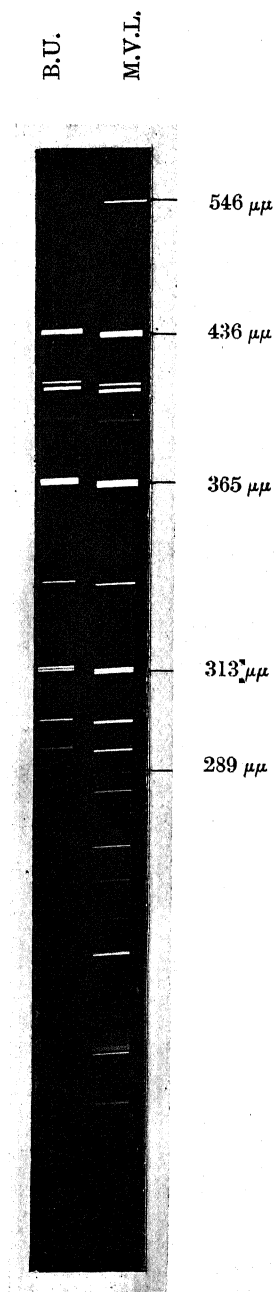


FIG. 2.—Comparison of spectra of Mercury-vapour Lamp (M.V.L.) and same through Blue Uviol Glass (B.U.). The visible part of the M.V.L. is cut short in the photograph. It extends to about $770\ \mu\mu$. The ultra-violet of the M.V.L. extends to about $223\ \mu\mu$. Time exposures (seconds) B.U. : M.V.L. = 3 : 1.

REFERENCES.

- (1) Kestner, Peemuller, Plaut, 'Klin. Therap. Wehnschr.,' vol. 44, p. 2018 (1923).
 - (2) Fries, 'Proc. Soc. Exp. Biol. and Med.,' vol. 22, p. 431 (1925).
 - (3) Krogh, 'The Respiratory Exchange of Animals and Man,' p. 102 (1916).
 - (4) Hausmann, H. Meyer's 'Lehrbuch der Strahlentherapie,' vol. 1, p. 671 (1925).
 - (5) Leonard Hill and Campbell, Hill's 'Sunshine and Open Air,' p. 114 (1924); 'Medical Research Council, Special Report No. 73,' p. 145 (1923); 'Brit. Med. J.,' vol. 1, pp. 301 and 385 (1922).
 - (6) Harris, 'Proc. Roy. Soc.,' B, vol. 98, p. 171 (1925).
 - (7) Cathcart, 'J. Roy. Army Med. Corps,' vol. 31, p. 339 (1918).
 - (8) Haldane, 'Respiration,' p. 21 (1922); Campbell, Douglas and Hobson, 'J. Physiol.,' vol. 48, p. 306 (1914).
 - (9) Pembrey, 'J. Physiol.,' vol. 15, p. 401 (1894); Macleod, 'Amer. J. Physiol.,' vol. 18, p. 1 (1907); Cramer, 'Report Imp. Cancer Res. Fund,' p. 427 (1908).
-

*A Study of Certain Forms of Inhibition and Acceleration
of Hæmolysis.*

By ERIC PONDER.

(Communicated by Sir E. Sharpey Schafer, F.R.S.—Received January 30, 1926.)

(From the Department of Physiology, Edinburgh University.)

In considering the kinetics of the simpler hæmolytic systems, we have already dealt at some length with one form of inhibition—that shown by serum in conjunction with saponin or one of the bile salts as a lysin (1, 2). This form of inhibition is that which has been most widely studied, and is one of great importance, for it has been shown that the inhibitory properties of serum are possessed by hæmoglobin, and that these properties must be taken into account when dealing with the percentage hæmolysis curves for such a substance as saponin (3). Other substances than serum, however, inhibit the action of saponin and the bile salts, and besides these, there is the class of substances which produce an acceleration of hæmolysis; no quantitative study of these has yet been made, and since we cannot proceed farther with the kinetics of simple hæmolytic systems without a knowledge of the way in which these accelerating and inhibiting substances produce their effect, this paper will be devoted to a quantitative study of some of the reactions involved.

The phenomena to be studied fall into two classes. In the first we have those substances which produce an inhibition of hæmolysis, such as certain bases and sugars. The inhibitory action of members of this group is quite different from that shown by serum. In the second group are those substances which accelerate hæmolysis, such as most acids. One other class of acceleration can be added—that produced by serum when added in a certain manner to a hæmolytic system containing one of the bile salts; this phenomenon, however, we do not propose to consider in this paper, as its investigation presents special difficulties.

Group 1.—Inhibitors.

Of the many substances which fall into this group, we shall consider only a few, leaving the observations to be extended to other cases.

1. *Sucrose*.—This substance, in suitable concentration, inhibits the action of saponin. In order to obtain quantitative results, we use the following technique :—

The curve showing the relation of the time taken for complete hæmolysis to the dilution of lysin acting is first obtained, the experiment being conducted, as are most of those in this paper, at 25° C. A series of dilutions of saponin in saline (0·85 per cent. NaCl) is prepared, each solution being 2·5 times as concentrated as finally desired. Of each dilution, 0·8 c.c. is mixed with 0·8 c.c. of saline, the mixture warmed to 25° C., and 0·4 c.c. of cell suspension added. The cell suspension consists of the cells from 1 c.c. of human blood, thrice washed with saline after being received into citrate, and finally suspended in 20 c.c. of saline. The curve for saponin acting on the cells alone passes through the following points :—(20,000, 0·75), (30,000, 1·75), (40,000, 3·4), (50,000, 5·6), (60,000, 10).

The curve for the lysin acting on the cells in the presence of 2 per cent. sucrose is next obtained by substituting for the 0·8 c.c. of saline added to each of the dilutions of saponin as above, 0·8 c.c. of a 5 per cent. sucrose solution in 0·425 per cent. saline. This concentration of saline is necessary in order to maintain the correct tonicity in the hæmolytic system. This curve passes through the points (20,000, 1·35), (30,000, 3·1), (40,000, 5·5), (50,000, 11).

Following the procedures employed in (1), the following table is now drawn up. Here δ_1 is the dilution of lysin added and c_1 its corresponding concentration, that is, the number of milligrammes of lysin actually added to the tube containing the cells. The dilution of lysin which, acting alone, would take the same time for complete hæmolysis as is observed with the lysin together with the

inhibitory agent is δ_2 , and its corresponding concentration c_2 . The difference between c_1 and c_2 is x .

δ_1 .	c_1 .	δ_2 .	c_2 .	x .
20,000	0.100	27,000	0.074	0.026
30,000	0.066	33,000	0.052	0.014
40,000	0.050	50,000	0.040	0.010
50,000	0.040	61,000	0.033	0.007

Plotting c_2 against x , it will be seen that the points lie on a straight line. We have, therefore, a different relation between the variable from that obtained when serum is the inhibitory agent, for under these circumstances the points lie on a curve (1, 2). Since $x = (c_1 - c_2)$, we may now plot c_1 against c_2 , when we shall get another straight line, which makes an intercept on the c_2 -axis. The equation of this line is $c_1 = R(c_2 - k)$, where $R = 1.46$, and $k = 0.005$; this is the general expression which will be shown to be applicable to a large number of hæmolytic systems in which there is acceleration or inhibition.

Supposing that k were zero, the meaning of this expression would be that, in the hæmolytic system containing sucrose, a concentration of lysin c_1 produces hæmolysis as if it were of concentration c_1/R , and acting on cells in the absence of sucrose, or, what amounts to the same thing, that the addition of the inhibitory substance has the effect of increasing the resistance of the cells to the lysin R times. It is not possible, at this stage of the inquiry, to say that the action of the inhibitory substance is on the cells themselves, increasing their resistance; it is equally possible that the sucrose affects the activity of the lysin, or that the process by which the lysin combines with the cells may be affected. The best way to regard the finding is to look upon R as measuring the resistance of the cells in the cell-saponin-sucrose system as compared with that in the cell-saponin system, without specifying which component of the system is affected by the addition of the sucrose.

The meaning of the constant k is less clear. In the study of the inhibition produced by serum a similar constant appeared, and the meaning attached to it was that a certain small portion of the lysin combines with the cells in such a way as to be unaffected by the presence of the inhibitory agent, which reacts with the remaining portion of the lysin only. In this instance a similar explanation would suffice, but it is better in the meantime to regard k as an empirical constant, and to leave its meaning undefined. We propose to make a special study of the matter.

The next point of interest is to inquire whether the value of R and k as obtained above are the same at all temperatures of experiment. The curve for saponin acting alone at 35°C . is therefore obtained, and is as follows:— (30,000, 0.6), (40,000, 1.25), (50,000, 2.6), (60,000, 4.5), (70,000, 10), (80,000, 25). The curve for saponin acting with 2 per cent. sucrose at 35°C . is:— (30,000, 1.2), (40,000, 2.6), (50,000, 5.5), (60,000, 13). From these curves the following figures are obtained:—

δ_1 .	c_1 .	δ_2 .	c_2 .
30,000	0.066	39,000	0.051
40,000	0.050	50,000	0.040
50,000	0.040	62,000	0.0322
60,000	0.033	73,000	0.0274

Plotting c_1 against c_2 , we obtain the line $c_1 = R(c_2 - k)$, where, as in the experiment conducted at 25°C ., $R = 1.46$ and $k = 0.005$. These values of the two constants are therefore the same at the two temperatures.

Proceeding in a similar way to the above, we have investigated the inhibitory effect of concentrations of sucrose other than 2 per cent. In these cases it is important to dissolve the sugar in such a concentration of NaCl as will give a correct tonicity for the mixture, for the sucrose exerts an osmotic pressure of its own. This being done, the following values of R and k were obtained for different concentrations of the inhibitory agent:—

Sucrose concentration, per cent.	R .	k .
1.0	1.25	0.005
2.0	1.46	0.005
3.0	1.51	0.005
4.0	1.57	0.005

In all these cases it is to be noted that the concentrations are those in the 2 c.c. of the hæmolytic system, which results from the mixture of the sucrose solution, the lysin and the cells. It will be observed that as the concentration of sucrose increases, the value of R increases also, but not in simple proportion, and that, so far as the findings of the method show, k is constant. The method is, however, not such as would detect slight variations in this latter constant.

In treating the curve which results from the plotting of c_1 against c_2 as a straight line, we have considered the possibility of its being really a part of

a very flat curve passing through the origin. The most careful experiments have, however, failed to reveal sufficient variation from a straight line to warrant us abandoning this convenient curve, which describes the results within the experimental range when extended as much as possible. We feel, nevertheless, that any extrapolation should be employed with caution, especially if it be in the direction of the origin.

2. *Sodium Hydroxide*.—The addition of small quantities of NaOH is sufficient to inhibit lysis by saponin or the bile salts. In the case of sodium taurocholate, as little as N/5,000 NaOH produces the effect, which is unmeasurable by these methods.

With saponin and N/1250 NaOH, the results are similar to those obtained with sucrose. Plotting c_1 against c_2 gives a straight line with $R = 1.5$ and $k = 0.008$. The pH of such a solution was found by potentiometer measurement to be 9.90.

3. *Arginine*.—In the course of an examination of a number of amino-acids for inhibition and acceleration effects, arginine was found to inhibit lytic action of saponin and of sodium taurocholate. A solution of this amino-acid in saline is alkaline, and the inhibition may therefore be compared with that of NaOH.

Saponin acting with N/1250 NaOH and saponin acting with 1 in 1250 arginine were found to give exactly the same curve, the plotting of c_1 against c_2 giving the same straight line with $R = 1.5$ and $k = 0.008$. As regards their inhibitory effect, these two substances, in these concentrations, are identical. As regards their pH, however, they are by no means the same, for, while the pH of the NaOH was found to be 9.90, that of the arginine solution giving the same inhibition was found to be 8.94. It must therefore be concluded that the pH alone is not responsible for the inhibition.

In view of certain finding to be recorded below, an important point should here be noted. If the arginine be allowed to remain in contact with the cells for some time before the lysin is added, the resulting inhibition is very much greater than if the lysin and the arginine be mixed and the cells added. This is not in any way surprising, but it is rather remarkable that under these circumstances the degree of inhibition tends to be independent of the amount of arginine used as an inhibitor. Arginine 1 in 600, 1 in 2500, and 1 in 4000 all gave the same amount of inhibition if the cells were left in contact with these concentrations for about five minutes before the addition of the saponin. When the experiment is performed with the additions in this order, it is not

possible to produce a greater inhibition than is exerted by one of these concentrations; the full effect seems to be attained by such a low concentration as 1 in 4000, below which the inhibition fails. We are unable to explain this result, and merely note it in passing. A similar result has been obtained for several other cases of inhibition, the effect appearing to be independent of the concentration of inhibitory substance over quite a considerable range.

These are only three of many substances which produce inhibition and whose action is described by a linear expression. We intend to deal with the inhibitory action of the sugars, and also with that of certain salts, in other papers, as new points arise in these connections; it may, however, be stated here that these inhibitions are similar in a general way to those discussed above. It may also be mentioned that we have examined the following substances with respect to their effect on hæmolysis, and have found them inert: glycine, alanine, phenyl-alanine, valine, cystine, taurine, guanidine, leucine, and sarcosine. Guanidine is stated by Secker (4) to alter the permeability of the erythrocyte if calcium is present; we have not found it to affect hæmolysis as either an accelerator or an inhibitor. This finding does not, of course, necessarily conflict with that of Secker.

Group 2.—Accelerators.

Acceleration of hæmolysis is produced by most acid substances, some of which we shall consider here.

1. *Acetic Acid*.—The investigation of the acceleration produced by this, or any other, substance is similar to the investigation of an inhibition. The curve for the lysin acting alone is first plotted, and thereafter the curve for the lysin acting with the added accelerator.

With N/500 acetic acid acting with saponin we get the following curve: (40,000, 0·5), (50,000, 0·75), (60,000, 1·1), (70,000, 1·75), (80,000, 2·3). Saponin acting alone gives: (20,000, 0·75), (30,000, 1·2), (40,000, 2·3), (50,000, 3·5), (60,000, 6), (65,000, 11), (70,000, 20). From the two curves we get the following figures. Here $x = (c_2 - c_1)$.

δ_1 .	c_1 .	δ_2 .	c_2 .	x .
40,000	0·050	15,000	0·133	0·073
50,000	0·040	20,000	0·100	0·060
60,000	0·033	28,000	0·071	0·038
70,000	0·028	34,000	0·058	0·030
80,000	0·025	40,000	0·050	0·025

Plotting x against c_2 , a straight line results, as in the case of an inhibition. Plotting c_1 against c_2 , we get another straight line, whose equation is $c_1 = R(c_2 + k)$, where $R = 0.29$ and $k = 0.039$. This is the same type of expression as is applicable to an inhibition; where inhibition occurs, R is greater than unity, and k is negative, while where acceleration occurs, R is less than unity and k is positive. The meaning to be attached to R in the case of an acceleration is the same as it bears in the case of inhibition; we regard in the meantime as an empirical constant. It is usually larger than in cases of inhibition, but this is quite misleading; if the constants are to be compared, the intercepts made by the lines obtained by plotting x against c_2 should be compared, rather than the values for k themselves. These intercepts are both positive, and do not differ greatly.

The pH of this N/500 acetic acid in saline was found to be 3.73 by potentiometer measurement.

2. *Glutaminic Acid*.—In concentration of 1 in 10,000, this amino-acid, acting with saponin, gave the following curve: (30,000, 0.4), (40,000, 0.75), (50,000, 0.95), (60,000, 1.2), (70,000, 2), (80,000, 3.5), (100,000, 9.5). The curve for saponin acting alone was the same as that given for saponin alone in the previous section. The relation of c_1 to c_2 is a line described by $c_1 = R(c_2 + k)$, where $R = 0.375$ and $k = 0.027$. This experiment, it should be noted, is one of the least satisfactory we have done, and the errors in it may be taken as being about as great as will be met with. The calculated values of c_1 , however, are as follows: 0.064, 0.047, 0.040, 0.035, 0.030, 0.025, and 0.021, instead of 0.066, 0.050, 0.040, 0.033, 0.0285, 0.025, and 0.020. These errors are small, and as a rule the experimental errors are smaller still.

3. *Histidine Monohydrochloride*.—This acid substance accelerates hæmolysis by saponin, and the relation between c_1 and c_2 is of the type described above. The values of the constants are $R = 0.52$, $k = 0.017$, when 1 in 2500 histidine is used.

In view of the fact that these accelerating substances are all acid, we have compared, in the case of histidine monohydrochloride and acetic acid, the pH and the accelerating action. Adding to 0.8 c.c. of 1 in 50,000 saponin 0.8 c.c. of the undernoted concentrations of the two accelerating agents, and thereafter 0.4 c.c. of cell suspension, the time taken for complete hæmolysis was noted in each case. The pH of the added accelerator was afterwards determined. The following table gives the results:—

Acetic acid.			Histidine.		
Concentration added.	Time mins.	pH.	Concentration added.	Time mins.	pH.
—	4.0	—	—	4.0	—
N/200	0.75	3.58	0.5 per cent.	1.2	4.22
N/300	0.85	3.62	0.25 "	1.4	4.44
N/400	0.95	3.66	0.10 "	1.6	4.52
N/500	1.5	3.73	0.05 "	2.5	4.69
N/1000	2.0	3.82	—	—	—

From these results it appears that it is not only the *pH* of the added accelerator which determines the amount of acceleration; the addition of 0.8 c.c. of acetic acid of *pH* 3.73 has nearly the same effect as the addition of the same quantity of histidine of *pH* 4.52. This is a result similar to that obtained in the case of the inhibition by the alkaline substances NaOH and arginine, where the inhibition was found to depend not on the *pH* only.

Histidine, and also histamine, which acts very like it, accelerate the hæmolysis by sodium glycocholate, and also by sodium taurocholate. In this respect they are similar to most acid substances. The effect on glycocholate hæmolysis has already been described (5), although in a non-quantitative way, for this lysin presents such a curious time-dilution curve as to preclude the application of the methods described in this paper. In the paper last quoted, an attempt was made to consider the acceleration due to histamine along with that shown by serum proteins when added to an existing hæmolytic system of bile salt and cells; further investigations have led us to think that the two phenomena, though similar at first sight, are quite distinct in their mechanism and are better dealt with separately. As was shown in that paper, the effect of adding acid to the solutions of histamine used as accelerators was to increase the acceleration, while the effect of added alkali was to diminish the acceleration; this is what might be expected, as the acceleration appears to be associated with the *pH*. It was also observed that there was a great disproportion between the effects produced by a 1 in 2000 histamine and those produced by a 1 in 5000 solution, a fact which is interesting in view of the present observations on the degree of inhibition due to different dilutions of arginine. It suggests that at the point where the disproportion occurs, a certain critical *pH* is passed, and that solutions of histamine more near to neutrality than this possess little or no accelerating action. In view of this later work, several of the hypotheses put forward in (4) to explain the results obtained by experi-

ment require revision, for it appears that the hæmolytic systems under consideration were more complex than was realised at the time; the experimental facts, however, for the most part stand unaltered.

4. *Aspartic Acid*.—This amino-acid behaves much like glutaminic acid, as an accelerator of lysis by saponin, the bile salts, and the soaps. Quantitative work must be confined to the effect on the action of the first lysin, for sodium taurocholate is precipitated by the acid, and the fine opalescence interferes with the end-point; the soaps likewise become opalescent, and no satisfactory results can be obtained with sodium glycocholate because of its curious time-dilution curve. With saponin and 1 in 2500 aspartic acid, we have obtained excellent values for the constants in the linear expression above described—indeed, this accelerator is one of the easiest to work with—the values being $R = 0.27$, $k = 0.015$, while with half the quantity of accelerator the values were $R = 0.35$, $k = 0.014$. Even with such a low concentration as 1 in 20,000 aspartic acid, acceleration can be observed, while concentrations greater than 1 in 2500 produce not only acceleration of saponin hæmolysis, but lysis of themselves. Experiments with such concentrations are, therefore, better avoided.

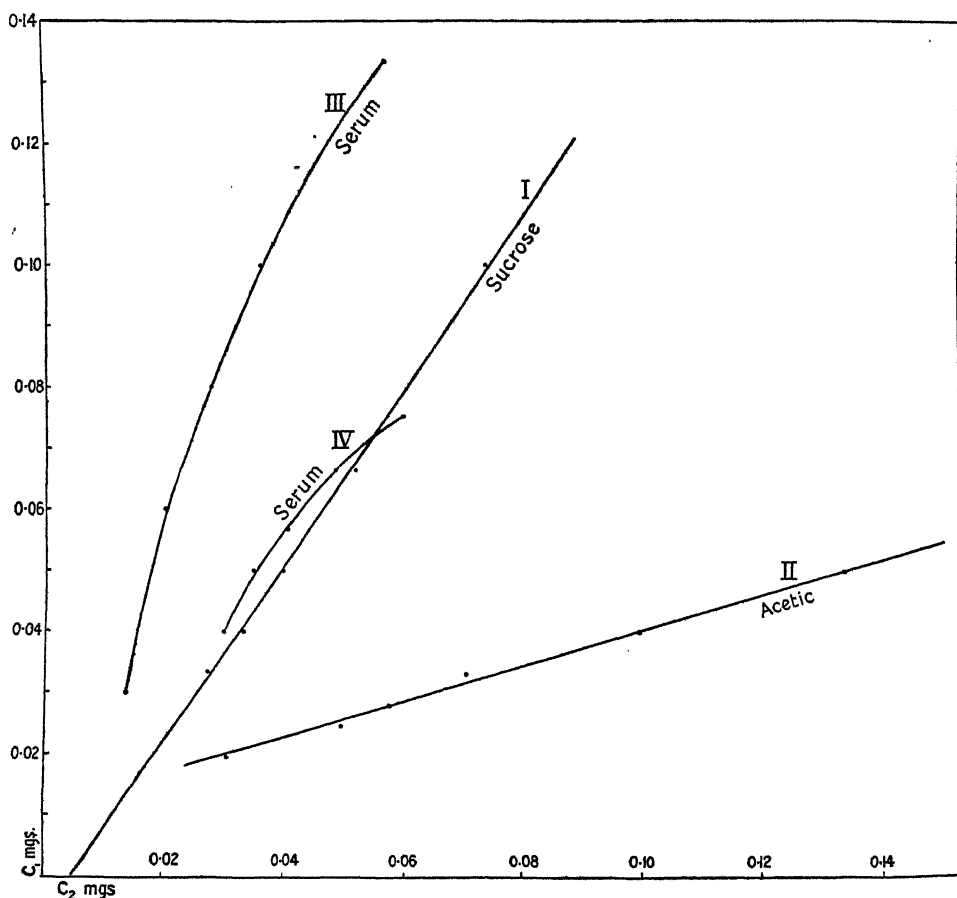
5. *Potassium Chloride*.—In most of the hæmolytic systems hitherto considered by us, the lysin and cells have been suspended in 0.85 per cent. NaCl. If some of the NaCl be replaced by another salt, the hæmolytic system may show an alteration; hæmolysis may occur either more rapidly or more slowly than in the first system. In the case of saponin and human cells, the replacement of Na by K results in an acceleration.

This may be investigated by plotting the time-dilution curves for the two systems: (1) saponin 0.8 c.c., 0.85 per cent. NaCl 0.8 c.c., cell suspension 0.4 c.c., and (2) saponin 0.8 c.c., M/6.8 KCl 0.8 c.c., cell suspension 0.4 c.c. The tonicity of the two systems is the same—an important point—but in the second two-fifths of the Na content of the first is replaced by K.

In a large number of experiments we have obtained the following values of R :—0.8, 0.75, 0.77, 0.81, 0.79, 0.83. In conjunction with these, values for k , lying between 0.003 and 0.006, have been obtained. The effect of the replacement of a part of the Na by K is thus fairly constant, although it varies a little with different cell suspensions. In this connection it may be pointed out that it is not to be assumed that because a certain quantity of K has a certain effect on the saponin lysis of human cells, that the same effect will be met with when the cells of some other animal are used. This supposition is a very common fallacy, and is responsible for a considerable amount of confusion in the

literature. Our experience of hæmolytic systems leads us to believe that it is never safe to argue from results in one system to those in another, however slight the difference between the systems may appear.

In order to show the type of graphical result obtained, the results of two of the experiments given above in full have been collected in the accompanying diagram, inspection of which will show how well the experimental points fall on the straight lines taken as describing the relations. To the figure two curves, obtained from an experiment in which the inhibitor was serum, have been added, for purposes of comparison.



- I. Sucrose, 2 per cent.—Inhibition.
- II. Acetic Acid, N/500—Acceleration.
- III. Serum, 0.02 c.c.—Inhibition.
- IV. Serum, 0.0025 c.c.—Inhibition.

The Mechanism of the Inhibition.

In the hæmolytic systems so far discussed there have been only three components—the cells, the lysin, and the added accelerator or inhibitor. The observations made on the action of the last component may now be amplified by the consideration of a method which indicates its mode of action. The essential point is to ascertain whether the accelerator or inhibitor acts on the lysin or on the cells; in the case of the inhibition produced by serum, for example, evidence has been brought forward to show that the lysin is affected, but it cannot be assumed that the action of the substances described in this paper is similar, and special methods must be used for the investigation of the matter.

The principle of the method used is an obvious one. The cells are allowed to remain in contact with the accelerator or inhibitor for a certain period under certain conditions, and are then brought into a hæmolytic system in which none of the accelerator or inhibitor is present. If in this system there occurs acceleration or inhibition, this is taken as evidence of the effect of the accelerator or inhibitor on the cells themselves; if the hæmolytic system is the same as one of which untreated cells form a component, it is to be taken that the cells are not directly affected. In practice, the procedure is carried out as in the following description, in which the action of arginine on saponin hæmolysis will be taken as an example to be worked out in detail.

1. *Arginine and Saponin.*—To 10 c.c. of 1 in 1000 arginine in saline is added 5 c.c. of the standard cell suspension. The mixture is allowed to remain at 25° C. for thirty minutes, occasional stirring being advisable; at the end of this time 10 c.c. of the mixture is transferred to a tared flask, great care being taken that the cells are uniformly distributed in the fluid when the transference is made. The fluid from the flask is distributed into two centrifuge tubes, each of 10 c.c. capacity, and 5 c.c. of saline added to each. The contents of the tubes are centrifuged, the supernatant fluid pipetted off, and the cells together with the small amount of fluid which remains replaced in the tared flask. The tubes are washed out with several small additions of saline to remove all the cells, and the washings added to the flask. The 10 c.c. volume of this flask is then made up with saline, the cells thoroughly distributed throughout the fluid, and the suspension used for plotting time-dilution curves.

Three curves are now plotted. The first is that of saponin acting on the untreated cell suspension. The second is obtained by adding to 0.8 c.c. of a series of saponin dilutions 1.2 c.c. of the mixture of cells and arginine which is

left over from the 15 c.c. of mixture after withdrawal of the amount needed to fill the 10 c.c. flask. The proportion of cells and arginine solution forming the mixture is such that this corresponds to mixing 0.8 c.c. of each saponin dilution with 0.8 c.c. of arginine solution and 0.4 c.c. of standard cell suspension. The third curve is obtained by mixing with a series of saponin dilutions 1.2 c.c. of the suspension prepared from the washed cells. In each of these systems the quantity of cells is the same; in the first the cells are untreated, in the second they are in the presence of arginine, in the third they have been treated with arginine, but this inhibitory agent has been washed away.

From the plotted curves there is determined the value of R for the hæmolytic system containing arginine, and the value of R_1 for that containing the cells which have been treated with arginine and subsequently washed. As an illustration, the figures for one experiment may be given.

Arginine-saponin-cell system.		Saponin-washed cell system.	
c_1 .	c_2 .	c_1 .	c_2 .
0.066	0.049	0.066	0.054
0.050	0.038	0.050	0.041
0.040	0.031	0.040	0.034
gives $R = 1.55$ $k = 0.005$		gives $R_1 = 1.4$ $k_1 = 0.005$	

In this experiment the concentration of the arginine added to the cells was 1 in 1000, and the concentration acting on the cells was therefore 1 in 1500.

This result shows that the effect of the arginine is on the cells themselves, for it persists after by far the greater part of the inhibitor has been removed by the washing and re-suspension of the treated cells. The whole of the arginine is not, however, removed by this one washing, and it is necessary for us to know how much of it remains in the suspension of the washed cells.

If a represents the quantity of fluid in which the cells are suspended before washing, and x_0 the concentration of inhibitor therein, the absolute amount of inhibitor is ax_0 . A quantity of washing fluid m is added, centrifuging carried out, and a quantity of fluid b left in contact with the cells. Then the amount of inhibitor in this fluid will be

$$x_1 = \frac{b}{a} \left(\frac{a}{m+a} \right) ax_0.$$

This will also be the amount of inhibitor in the suspension of washed cells.

In this case we have $a = 10$ c.c., and $x_0 = 1$ milligramme per cubic centimetre. The value of m is 10 c.c., and that of b may be estimated as not greater than 0.5 c.c. Hence $x_1 = 0.25$ milligramme—the amount of arginine in the fluid in which the cells are re-suspended, namely, 10 c.c. This gives the dilution of arginine in the washed cell suspension as 1 in 40,000, an amount which is negligible. So far as the inhibitory effect is concerned, it may therefore be taken that all the arginine is removed from the cells by the one washing. Any increased resistance of these washed cells is therefore due to some alteration produced in the cells during their 30-minute contact with the arginine.

The removal of the inhibitor from the hæmolytic system and the washing of the cells result, as the table shows, in a fall in the inhibition from a value of $R = 1.55$ to a value of $R_1 = 1.4$. To compare the inhibitory effects described by the two constants, we take the ratio $\frac{(1 - 1/R_1)}{(1 - 1/R)}$, an expression which is correct only if $k = k_1$. When this condition is not fulfilled, the comparison is best done graphically. In this case the ratio is 0.8, and we may therefore conclude that of the total inhibition produced by the arginine, 0.8 can be explained by the effect on the cells themselves, while the remainder, 0.2 of the total, is unaccounted for.

2. *Sucrose and Saponin*.—Proceeding in the same way, the effect of a 10 per cent. sucrose on the cells was determined. The cells were allowed to remain in contact with the sucrose for 30 minutes at 15° C., and the effect of saponin on the mixture determined. The cells were then washed and re-suspended, and the effect of saponin again obtained. Comparison of the curves with that for saponin acting alone in the absence of sucrose gave $R = 1.35$, $k = 0.004$, $R_1 = 1.3$, $k_1 = 0.004$. Of the total inhibition produced by the sucrose, whose concentration in the hæmolytic system was 4 per cent. (10 per cent. diluted 2.5 times), 0.9 can therefore be accounted for by an action of the sucrose on the cells, while 0.1 of the total disappears on the washing of the cells.

It will be noted that the value of R obtained in this experiment is smaller than that given earlier in this paper for the same concentration of sucrose, but where the sucrose and the lysin were first mixed at 25° C., and the cells added thereafter. The difference between the two results indicates that in this latter case the inhibition is only 0.72 of that in the instance previously mentioned; this may be due to the different methods of mixing the components of the hæmolytic system in the two cases, but is more probably due to differences in the condition of the cells in the two suspensions. This discrepancy between the values of this constant emphasises the fact that the value of R for a parti-

cular system is not invariable ; small variations in the figure occur, and the proper value must be determined on every occasion.

3. *Aspartic Acid and Saponin*.—The method outlined above can be applied equally well in the case of an accelerator. The accelerator is allowed to remain in contact with the cells in the absence of lysin for a short time, the cells then washed and re-suspended, and the effect of saponin upon them compared with the effect of that lysin on cells untreated with the accelerator. The following results were obtained for 1 in 3000 aspartic acid in contact with cells for 10 minutes at 15° C. :— $R = 0.31$, $k = 0.004$, $R_1 = 0.5$, $k_1 = 0.004$. This means that of the total inhibitory effect of the accelerator, only 0.46 remains after the washing and re-suspension of the cells—a comparatively small figure. The effect of the aspartic acid is therefore, partly at least, on the cells themselves, a fact which might have been foreseen from the lytic action of the acid when in higher concentrations.

5. *Glutaminic Acids and Saponin*.—In the case of glutaminic acid, acting on the cells for 10 minutes at 15° C. in concentration of 1 in 8,000, the following results were obtained for the acceleration before and after washing the cells : $R = 0.5$, $k = 0.006$, $R_1 = 0.63$, $k_1 = 0.006$. Of the total inhibition, only 0.58 remains after the washing of the cells, a small figure, as in the case of aspartic acid.

In these experiments it will usually be found that there is a little scope in selecting the particular line which describes the relation between c_1 and c_2 , as this line is obtained graphically, for the application of the method of least squares is a refinement which is not only unnecessary but which leads to a spurious accuracy. Under these circumstances it has been our practice to select, if possible, a line which will make $k = k_1$, a simplification of subsequent calculation being thereby produced. Such a procedure is quite justifiable under most circumstances, but must, of course, be used with care and discrimination.

In the same connection it may be observed that in experiments such as these, continual regard must be had to the extent of possible experimental errors. These errors vary in extent at different parts of the time-dilution curve, and an ability to assign to them their proper significance is therefore a matter of experience. It is, for example, a far worse error to observe 1.5 minutes as the time for complete hæmolysis instead of 1.25 minutes, than it is to observe 9 minutes instead of 7 minutes ; the former error seems the smaller, but that it is much more serious may be seen at once by translating it from terms of time into terms of milligrammes of lysin acting. Or again,

if by the addition of one substance the time for complete lysis is lengthened from 1 minute to 3 minutes, and by the addition of another substance it is lengthened to 15 minutes, the second inhibition is only 1.6 times the first, despite the great delay in hæmolysis.

Such facts as these make the expression of an acceleration or an inhibition in terms of time very misleading, and unfortunately this is the method generally used. The only satisfactory way is to eliminate the time factor in the expression of the results, so that these are given in terms of the quantity of lysin necessary to produce the results observed. This method of expressing results is, however, one which requires familiarity before its value is appreciated.

Summary.

This paper is concerned with the development of a method for dealing with hæmolytic systems which contain substances which produce acceleration or inhibition. Four types of such effect are recognised: (1) the inhibition produced by plasma and serum, described by expressions which suggest an interaction between the plasma or serum and the lysis, not unlike those which occur in adsorption processes; (2) the inhibition produced by certain bases, sugars and neutral salts, which are described by linear expressions; (3) the acceleration produced by acids and by certain salts, also described by linear expressions; and (4) the acceleration produced by plasma or serum when added to a hæmolytic system in which the lysin is one of the bile salts or soaps.

The first of these types has already been dealt with (1, 2), and this paper deals with types 2 and 3, which are the most generally met with. Type 4 remains to be considered.

With regard to types 2 and 3, the expressions necessary for their description are given, and the meaning of the constants therein considered. The object of the study being to investigate the kinetics of such hæmolytic systems, little attention has been paid to the possible ways in which the accelerating or inhibiting substances may bring about their effects, the only observation of note in this connection being that in the case of substances whose reaction is not a neutral one, the effect is not wholly dependent on the *pH*.

A development of the principal method is described, whereby the extent to which the accelerator or inhibitor can be considered as permanently affecting the cell component of the hæmolytic system may be ascertained. By these means it has been found that many of the substances of types 2 and 3 affect

the cells themselves, their action thus differing from inhibitions of type 1, which affect the lysin.

Little more has been done than indicate the very general applicability of these methods, and in subsequent papers it will be shown to what a variety of problems they apply.

The expenses of this research were defrayed by a grant from the Carnegie Trust for the Universities of Scotland.

REFERENCES.

- (1) Ponder, 'Roy. Soc. Proc.,' B, vol. 95, p. 42 (1923).
- (2) Ponder, 'Roy. Soc. Proc.,' B, vol. 98, p. 485 (1925).
- (3) Ponder, 'Roy. Soc. Proc.,' B, vol. 95, p. 383 (1923).
- (4) Secker, 'Journal of Physiology,' vol. 60, p. 286 (1925).
- (5) Ponder, 'Roy. Soc. Proc.,' B, vol. 93, p. 86 (1922).

The Isolation of Some Hitherto Undescribed Products of Hydrolysis of Proteins.—Part II.

By S. B. SCHRYVER, Professor of Biochemistry, and H. W. BUSTON, Research Assistant, Imperial College of Science and Technology.

(Communicated by Sir John B. Farmer, F.R.S.—Received February 4, 1926.)

The main object of the investigations described in this paper was the further development of the "carbamate process" for the separation of the hydrolysis products of proteins, already described by the authors,* and applied to the separation of the hydrolysis products of gelatin by Miss H. L. Kingston and Schryver.† It has been shown that, by this method, the hydrolysis products can be fractionated into the following main groups:—

I. Those products yielding barium salts insoluble in 70 per cent. alcohol; they consist entirely of dicarboxylic amino-acids.

II. Those products yielding barium carbamates insoluble in ice-cold water. Two products only have, up to the present, been found in this fraction,

* 'Biochem. J.,' vol. 15, p. 636 (1921).

† 'Biochem. J.,' vol. 18, p. 1070 (1924).

viz., glycine, and a base, hydroxylysine, isolated for the first time by the authors in conjunction with Dr. D. H. Mukherjee.*

III. Those products yielding barium carbamates soluble in ice-cold water. The free amino-acids from this fraction could be divided into two sub-fractions : (a) a basic sub-fraction precipitable by phosphotungstic acid ; (b) a non-basic sub-fraction, not so precipitable.

This last fraction (III, b) contains the largest number of the mono-amino-acids, including leucine, valine, alanine, phenylalanine, proline and oxyproline, and probably other substances. The results detailed below were obtained in the course of an investigation directed towards obtaining a systematic method for the separation of the constituents of this fraction.

Emil Fischer, many years ago, called attention to the probability of the existence of amino-acids, which at that time had not been isolated, and which still remained undiscovered when the authors commenced this work ; these he considered were likely to be found among the hydrolysis products of plant proteins. These remarks of Fischer have been emphasized by Prof. T. B. Osborne in his monograph. It was accordingly decided to carry out the investigations on fraction III (b) with products isolated from plant proteins.

In the first experiments the fraction was obtained from the alkali-soluble protein of oats and was prepared by Mr. D. I. Evans, to whom the authors are indebted for the first materials used. The preliminary investigations revealed the existence in this fraction of certain amino-acids apparently unlike in properties any of those hitherto described. The amounts present were small, the nitrogen contained in them amounting to not more than about 4 per cent. of the total nitrogen, and it was found necessary to prepare them again from a relatively large amount of protein.

The methods for preparing oat proteins as described in the literature are extremely difficult to carry out, and by using them it would have taken a very long time to prepare sufficient quantities for the separation in reasonable amounts of the above-mentioned undetermined products. A modified method was therefore evolved which is described below. By this process some 750 g. of one protein were obtained from 18 kg. of oatmeal in the course of three or four weeks.

Preparation of the Oat Protein (Glutelin).

Fine oatmeal, in several batches each of 300 g., was made into a thin paste with water, and stirred into 2,000 c.c. of 0.4 per cent. caustic soda ; the mixture

* 'Roy. Soc. Proc.,' B, vol. 98, p. 58 (1925).

was allowed to stand for about two hours, after which period the starch in the oatmeal had become gelatinized. Hydrochloric acid was carefully added to the mixture till the pH was restored to about 7, as indicated by bromo-thymol blue. A small amount of taka-diasatase was well stirred in, together with a little toluene, and the whole kept for 24 hours at 37° in an incubator. By this time, the product had attained the consistency of a thin liquid, the starch having been hydrolysed. A little caustic soda was added to dissolve any separated protein, and the liquid filtered, first through muslin to remove coarse material, and then through a Sharples centrifuge. A pale brownish, opalescent filtrate was thus obtained, from which a copious white precipitate was produced on the addition of hydrochloric acid. This precipitate of crude protein was removed on the Sharples centrifuge, washed thoroughly with water, and then extracted for some hours with boiling 70 per cent. alcohol, to remove gliadin-like proteins. After this, it was again washed with water and extracted with 10 per cent. sodium chloride to remove nucleic acid (method of Clarke and Schryver).

The protein was finally purified by dissolving in 0·2 per cent. caustic soda, filtering through paper-pulp and reprecipitating by dilute acid. It was dried by washing with graded strengths of alcohol and finally with ether. In all, 18 kg. of oatmeal yielded 730 g. of dry protein, containing 14·4 per cent. nitrogen.

The distribution of nitrogen in this product, as determined by Mr. D. I. Evans, was :—

	Per cent. of total N.
Humin nitrogen	6·90
Amide	12·31
Diamino	21·91
Non-basic	58·98
Including—	
Non-amino N.	11·04

The protein was hydrolysed with sulphuric acid, and the various fractions were separated by the usual "carbamate method," details of which are given in the previous papers of the authors and their collaborators.

Investigation of the Monoamino Acids (Fraction III b).

The particular fraction to which attention was confined was, as already stated, that designated III b. The free acids were obtained from this in the usual way, the solution of them was evaporated to a syrup, and thrown into

alcohol. The solid obtained was extracted repeatedly with absolute alcohol in order to remove proline; the creamy white powder remaining, weighing when dry some 200 g., contained 10.8 per cent. nitrogen, and only a trace of ash. Attempts to fractionate this mixture further come under three main heads:—

- (a) by means of the picrates;
- (b) by precipitation of the methylene-imino compounds of the acids by basic lead acetate;
- (c) by means of the zinc (or cadmium) salts of the acids.

Method (a), the use of the picrates led to no convenient method of separation. The picrates were always obtained as a viscous syrup which dried to a hard mass, yielding nothing definite on extraction with various solvents.

Method (b), precipitation with basic lead acetate in the presence of formalin, certainly led to the separation of leucine, in part. The method consisted in adding, to a fairly strong solution of the amino-acids, excess of formalin and a strong solution of basic lead acetate, so long as a precipitate formed. From this precipitate, leucine was isolated and identified. After removal of the first precipitate it was found that a further large precipitate was formed on standing for 24 hours.

In using this method, the obtaining of the maximum precipitation presented some difficulty. The amount produced depended on the concentration of formaldehyde present, rising to a maximum with addition of more formalin, and then falling again as excess of formalin redissolved the precipitate first formed. On account of these difficulties of manipulation the experiments were not continued further.

Method (c), fractionation of the zinc salts of the amino-acids, led to a definite separation of some of the amino-acids present in the mixture. The method was as follows: The mixed amino-acids were dissolved in twelve times their weight of water, excess of freshly prepared zinc hydroxide was added and the liquid was boiled under a reflux condenser for about half an hour. The excess of zinc hydroxide was removed, washed and dried; the filtrate, on evaporating on the steam-bath, formed crystalline crusts, which were removed before the solution had evaporated to dryness. Finally, the liquid dried completely on the steam-bath to a pale yellowish syrup, containing a certain amount of crystalline matter; on taking up with a little water, the crystals remained undissolved and were added to the crusts previously separated. The solution remaining was evaporated to a syrup and precipitated by alcohol; the major part

was thereby obtained as a dry powder showing little tendency to become sticky in the air. The alcoholic liquid was repeatedly evaporated to dryness and redissolved in alcohol till finally a product was obtained which dissolved in 98-99 per cent. alcohol without residue.

To summarize, then, the following fractions of zinc salts were obtained :—

- (i) Insoluble zinc salts (with excess of zinc hydroxide).
- (ii) Sparingly soluble zinc salts.
- (iii) Zinc salts readily soluble in water, but precipitated by alcohol.
- (iv) Zinc salts soluble both in water and alcohol.

The total nitrogen present in the fraction III *b* was distributed among the zinc salt fractions, thus :—

	Per cent. of Total.
Zinc salt (i).....	2·5
„ (ii).....	26·9
„ (iii).....	47·8
„ (iv).....	20·4

Each of these four fractions was decomposed separately with hydrogen sulphide, the free acid obtained being washed with alcohol and dried to constant weight at 100° *in vacuo* over phosphorus pentoxide. Estimation of nitrogen in each fraction by Kjeldahl gave :—

Acids from zinc salt :—

- (i) N = 10·11 per cent.
- (ii) N = 10·51 per cent., 10·58 per cent.
- (iii) N = 12·41 per cent., 12·32 per cent.
- (iv) N = 11·11 per cent.

These figures alone do not give much indication of the nature of each fraction, but they do indicate that, to some extent, a separation has been effected.

A parallel series of experiments, using cadmium salts in place of zinc, gave a similar result, but here apparently the separation was less complete. The nitrogen percentage in the free acids varied from 10·10 per cent. in (i) to 11·71 per cent. in (iii).

An attempt was also made to purify the individual fractions further by the formalin—basic lead acetate method referred to above. Fractions (i) and (ii) were almost completely precipitated at once; fraction (iii) showed only a small precipitate after standing for 24 hours; fraction (iv) gave little imme-

diate precipitate, but was found to be almost entirely precipitated after 24 hours. These experiments were not followed up, but, combined with those made originally with the lead acetate-formalin method, they are suggestive.

Investigation of the Four Fractions Obtained from the Zinc Salts.

Fraction (i).—This product, which was only recovered from the excess of zinc hydroxide in very small amount, was not dealt with. Its nitrogen content given above, suggests a mixture of leucine (which it resembles in appearance and behaviour) and phenylalanine.

Fraction (ii).—This fraction is somewhat sparingly soluble in cold water, and readily separates from a hot solution on evaporating in the pure white flakes characteristic of leucine. The nitrogen value given by Kjeldahl also suggests it to be fairly pure leucine. On combustion it gave the following figures :—

(i) 0.1132 g. gave 0.2270 g. CO_2 and 0.1027 g. H_2O .

(ii) 0.0970 g. gave 0.1951 g. CO_2 and 0.0870 g. H_2O .

Found :—C = 54.70 per cent., 54.81 per cent.

H = 10.08 per cent., 9.96 per cent.

N = 10.55 per cent., 10.58 per cent. (Kjeldahl).

Calculated for leucine ($\text{C}_6\text{H}_{13}\text{O}_2\text{N}$) :—

C = 54.97 per cent., H = 9.99 per cent., N = 10.69 per cent.

There seems little doubt that this fraction contains only leucine. The fact that leucine gives a sparingly soluble zinc salt has, of course, been known for a long time.

Fraction (iii).—This, judging by the nitrogen content, and also by the figures obtained by combustion, appears to contain a number of amino-acids. No further attempts at separation have so far been made. The figures obtained for the analyses were :—

(i) 0.1306 g. gave 0.2283 g. CO_2 and 0.0985 g. H_2O .

(ii) 0.0847 g. gave 0.1439 g. CO_2 and 0.0669 g. H_2O .

Found :—

C = 47.67 per cent., 47.49 per cent.

H = 8.38 per cent., 8.99 per cent.

N = 12.32 per cent., 12.41 per cent. (Kjeldahl).

These figures suggest a mixture containing valine (C = 51.24 per cent., H = 9.47 per cent., N = 11.96 per cent.), and alanine (C = 40.42 per cent.,

H = 7.93 per cent., N = 15.73 per cent.), but other amino-acids are certainly present.

Fraction (iv).—This again was obviously a mixture, and a method of sub-fractionation was found, based on the fact that one of the constituent amino-acids present gave a copper salt insoluble in methyl alcohol. The mixed copper salts, prepared as usual by boiling the amino-acid solution with excess of fresh copper hydroxide, were carefully dried and powdered, and extracted in the cold with dried and fractionated methyl alcohol, till the extract was no longer blue. The insoluble residue was recrystallized from hot water, readily yielding deep violet-blue crystals (short prisms), which on exposure to the air for some time showed slight efflorescence, and on desiccation gave a grey-blue powder. The alcohol-soluble salt did not readily yield crystals from aqueous solution, being very soluble in water and only giving an indefinite crystalline mass on evaporation.

The free acid was liberated in each case by means of hydrogen sulphide. That from the soluble copper salt was again reconverted into its zinc salt, this being dissolved when quite dry in absolute alcohol, the very small insoluble residue being thus removed. The free acid was again prepared from this zinc salt and both acids were dried *in vacuo* over phosphorus pentoxide. Of the acid yielding the insoluble copper salt the final amount obtained was 8.2 g.; of the other 18.1 g.

Acid from the Insoluble Copper Salt.—The free acid formed white crystalline plates, moderately soluble in cold water, readily soluble in hot water, and insoluble in alcohol. The acid was optically inactive, as is usual with acids obtained by the carbamate process. Nitrogen was found by Kjeldahl to be 11.64 per cent. An estimation by Pregl's micro-method (micro-Dumas) gave N = 11.71 per cent. Several combustions were carried out, on products, recrystallized from water, both by the usual method and by the micro-method of Pregl; these may be summarized as follows:—

(i) 0.1168 g. gave 0.1721 g. CO₂ and 0.0810 g. H₂O.

(ii) 0.0642 g. gave 0.0952 g. CO₂ and 0.0442 g. H₂O.

(iii) 0.003638 g. gave 0.005346 g. CO₂ and 0.002516 g. H₂O.

Found: C = 40.17 per cent., 40.44 per cent., 40.07 per cent.

H = 7.70 per cent., 7.62 per cent., 7.68 per cent.

N = 11.64 per cent. (Kjeldahl), 11.71 per cent. (micro-Dumas).

These figures agree with the compound of empirical formula C₄H₉O₃N, which requires C = 40.34 per cent., H = 7.56 per cent., N = 11.77 per cent.

An estimation of the carbon and hydrogen in the anhydrous copper salt gave figures in agreement with this, as shown below :—

0.004055 g. Cu salt gave 0.004791 g. CO_2 and 0.002052 g. H_2O .

Found : C = 32.22 per cent., H = 5.62 per cent.; calculated for $(\text{C}_4\text{H}_8\text{O}_3\text{N})_2\text{Cu}$, C = 32.55 per cent., H = 5.42 per cent.

A determination of the equivalent by the Sørensen method of titration with alkali in the presence of formalin, gave the following result :—

0.1205 g. of acid required 10.30 c.c. 0.1 N caustic soda giving an equivalent weight = 117.

The equivalent was also determined by estimation of copper in the copper salt (using the anhydrous salt). The figures obtained were :—

0.004055 g. anhydrous Cu salt gave, on combustion in oxygen, 0.001060 g. CuO , equivalent to 0.000847 g. Cu. Hence equivalent = 119.2.

[The hydrated salt, separating from aqueous solution, and dried at ordinary temperature and pressure, loses 10.7 per cent. of its weight in giving the anhydrous salt. It therefore contains apparently $2\text{H}_2\text{O}$ in the molecule, *i.e.* $\text{Cu}(\text{C}_4\text{H}_8\text{O}_3\text{N})_2 \cdot 2\text{H}_2\text{O}$, this requiring a loss of 10.81 per cent. on dehydration.]

A determination of amino-nitrogen by the van Slyke method showed that the whole of the nitrogen was in the amino form.

A determination of the molecular weight of the acid by the cryoscopic method in aqueous solution gave :

0.3342 g. dissolved in 50 c.c. of water gave a depression of 0.102°C .

Therefore, molecular weight = 121.7.

The above data would suggest that the product was a mono-carboxylic mono-amino acid of the formula $\text{C}_4\text{H}_9\text{O}_3\text{N}$, *i.e.* hydroxyaminobutyric acid, the next higher homologue of the well-known amino-acid, serine (hydroxyaminopropionic acid). In order to test this further, the benzoyl and the phenylisocyanate derivatives were prepared.

(a) Benzoyl derivative. This was made in the usual way by shaking one gram of the amino-acid with about six grams of benzoyl chloride and an equivalent amount of caustic soda, added in small quantities at a time. The liquid was filtered and the benzoyl compound precipitated, together with benzoic acid, by acidifying with sulphuric acid. The precipitate was filtered at the pump and washed repeatedly with large amounts of warm water (50° – 60°C .) to remove the benzoic acid. The residue was recrystallized from ether, when it formed fine white needles, insoluble in water and light petroleum, but soluble in ether, alcohol, etc. The m.p. of the crystals was 112° .

Carbon and hydrogen were estimated by micro-combustion, and nitrogen by micro-Dumas :—

(i) 0.003720 g. gave 0.009031 g. CO_2 and 0.001748 g. H_2O .

(ii) 0.004197 g. gave 0.145 c.c. nitrogen at 13°C . and 759.5 mm.

Found : C = 66.20 per cent., H = 5.22 per cent., N = 4.16 per cent.

Calculated for

$\text{C}_4\text{H}_8\text{O}_3\text{N}(\text{C}_6\text{H}_5\text{CO})$:—C = 59.20 per cent., H = 5.83 per cent., N = 6.27 per cent.

$\text{C}_4\text{H}_7\text{O}_3\text{N}(\text{C}_6\text{H}_5\text{CO})_2$:—C = 65.92 per cent., H = 5.19 per cent., N = 4.28 per cent.

The derivative would thus appear to be the dibenzoyl compound, as one would expect from a hydroxyamino acid.

(b) Phenylisocyanate derivative. This was obtained by shaking up 0.7 g. of the acid with 1 c.c. of phenylisocyanate and 15 c.c. of normal caustic soda. After filtering, the derivative was precipitated with dilute acid and removed after standing for some hours in the cold. It was recrystallized twice by dissolving in a very little absolute alcohol and adding excess of hot water ; on standing for some hours in the ice-chest, a deposit of fine crystals was found at the bottom of the liquid. These were removed and dried. They were insoluble in water, but easily soluble in ether and alcohol. Their m.p. was found to be 143°C ., but darkening began to occur at 120°C .

Carbon, hydrogen, and nitrogen were estimated by micro-methods —

(i) 0.002212 g. gave 0.004485 g. CO_2 and 0.001329 g. H_2O .

(ii) 0.002954 g. gave 0.006006 g. CO_2 and 0.001602 g. H_2O .

(iii) 0.002473 g. gave 0.248 c.c. nitrogen at 19° and 768 mm.

(iv) 0.003744 g. gave 0.378 c.c. nitrogen at 16° and 759 mm.

Found : C = 55.29 per cent., 55.41 per cent.

H = 6.01 per cent., 6.02 per cent.

N = 11.94 per cent., 12.01 per cent.

The phenylisocyanate of hydroxyaminobutyric acid would have a formula $\text{C}_{11}\text{H}_{14}\text{O}_4\text{N}_2$, which requires C = 55.46 per cent., H = 5.88 per cent., N = 11.77 per cent. It seems, therefore, to be established that this amino-acid is a hydroxyaminobutyric acid, $\text{C}_3\text{H}_5(\text{OH})\text{NH}_2\text{COOH}$. The position of the hydroxyl and amino groups is not established.

Some months ago, Gortner and Hofmann* isolated from the protein teozein, by precipitation of the hydrolysis products with phosphotungstic acid at -15°C ., an amino-acid which they considered was possibly of this constitution. The product isolated by them contained 28 per cent. of ash, and, allowing for this, was found to contain $\text{C} = 39.93$ per cent., $\text{H} = 9.09$ per cent., and $\text{N} = 12.00$ per cent. They prepared the phenylisocyanate, and quote one analysis of this, viz., $\text{C} = 55.40$ per cent., $\text{H} = 5.25$ per cent., $\text{N} = 11.93$ per cent. This derivative had a m.p. $= 141^{\circ}\text{C}$. These analyses do not agree very closely with those required by hydroxyaminobutyric acid. It may further be pointed out that the values calculated by these workers for the C and H content correspond with the formula $\text{C}_{11}\text{H}_{13}\text{O}_4\text{N}_2$, and not, as they state, with $\text{C}_{11}\text{H}_{14}\text{O}_4\text{N}_2$, and that the formula of hydroxyaminobutyric acid is repeatedly given as $\text{C}_4\text{H}_{11}\text{O}_3\text{N}$, instead of $\text{C}_4\text{H}_9\text{O}_3\text{N}$.

Acid from the Soluble Copper Salt.—This product was similar in appearance and behaviour to that described above, but was rather more soluble in cold water. Nitrogen was found by Kjeldahl to be 10.40 per cent., and by micro-Dumas, 10.61 per cent., being again wholly in the amino form. The equivalent of the acid was determined by Sørensen titration, and the molecular weight by the cryoscopic method :—

0.3480 g. required 26.0 c.c., 0.1 N caustic soda, giving equivalent $= 133.8$.

0.2756 g. in 50 c.c. of water gave a depression of freezing point $= 0.076^{\circ}\text{C}$.

Hence molecular weight $= 134.9$.

Combustions, both on the ordinary scale and by the Pregl micro-methods were carried out, giving :—

- (i) 0.0775 g. gave 0.1277 g. CO_2 and 0.0566 g. H_2O .
- (ii) 0.0869 g. gave 0.1437 g. CO_2 and 0.0645 g. H_2O .
- (iii) 0.003912 g. gave 0.006846 g. CO_2 and 0.002972 g. H_2O .

Found : $\text{C} = 44.93$ per cent., 45.11 per cent., 45.22 per cent.

$\text{H} = 8.13$ per cent., 8.25 per cent., 8.44 per cent.

$\text{N} = 10.40$ per cent., 10.61 per cent.

These figures give an empirical formula of $\text{C}_5\text{H}_{11}\text{O}_3\text{N}$, which requires $\text{C} = 45.11$, $\text{H} = 8.27$, $\text{N} = 10.53$ per cent.

The benzoyl and phenylisocyanate derivatives were prepared as in the case of hydroxyaminobutyric acid, and by the same methods. The former was similar in properties to that described above; it was slightly soluble in

* 'J. Amer. Chem. Soc.,' vol. 47, p. 580 (1925).

petroleum ether. The m.p. was found = 117°C . The following figures were obtained on analysis :—

- (i) 0.003318 g. gave 0.008155 g. CO_2 and 0.001627 g. H_2O .
- (ii) 0.004179 g. gave 0.141 c.c. nitrogen at 14°C . and 759 mm.

Found : C = 67.04 per cent., H = 5.45 per cent., N = 4.05 per cent.

The dibenzoyl compound $\text{C}_5\text{H}_9\text{O}_3\text{N}(\text{C}_6\text{H}_5\text{CO})_2$ requires :—

C = 66.86 per cent., H = 5.57 per cent., N = 4.11 per cent.

The phenylisocyanate melted at 145°C ., and gave the following figures on analysis :—

- (i) 0.003588 g. gave 0.007491 g. CO_2 and 0.002140 g. H_2O .
- (ii) 0.004560 g. gave 0.009526 g. CO_2 and 0.002739 g. H_2O .
- (iii) 0.003316 g. gave 0.314 c.c. nitrogen at 20°C ., and 768 mm.

Found : C = 56.92 per cent., 56.98 per cent. ; H = 6.58 per cent. ; 6.67 per cent., N = 11.24 per cent.

The phenylisocyanate of $\text{C}_5\text{H}_{11}\text{O}_3\text{N}$, having the formula $\text{C}_{12}\text{H}_{16}\text{O}_4\text{N}_2$, requires C = 57.15 per cent., H = 6.35 per cent., N = 11.11 per cent.

These analyses of the free acid and its two derivatives establish it to be a hydroxyamino acid, of the formula $\text{C}_5\text{H}_{11}\text{O}_3\text{N}$, corresponding with hydroxy-valine, $\text{C}_4\text{H}_7(\text{OH})\text{NH}_2\text{COOH}$. The position of the substituent groups has yet to be determined.

There seems to be evidence of the existence of other undescribed substances amongst the hydrolysis products of this oat protein. The authors have obtained, in appreciable quantity, a strongly basic substance which is readily soluble in alcohol, and does not apparently correspond with any of the usual hydrolysis products.

Summary.

From the hydrolysis products of one of the oat proteins (of which a new method of preparation is given) two hitherto undescribed amino-acids have been obtained. They were isolated by means of the authors' "carbamate method" and were found in the non-basic fraction of the soluble barium carbamates. These latter can, by means of the zinc salts, be separated into three main fractions : (a) a fraction (zinc salts) only slightly soluble in cold water, and yielding only leucine ; (b) a fraction easily soluble in cold water, but insoluble in alcohol, yielding chiefly alanine and valine ; (c) a fraction soluble in both cold water and alcohol.

The last named contained two amino-acids, which could be separated by taking advantage of the fact that one gave a copper salt insoluble in methyl alcohol, and the other a copper salt soluble in this solvent. The former has a formula corresponding with hydroxyaminobutyric acid $C_4H_9O_3N$, and gave a dibenzoyl compound, m.p. 112° , and a phenylisocyanate compound, m.p. 143° . The other amino-acid has a formula corresponding with hydroxy-valine $C_5H_{11}O_3N$, and gave a dibenzoyl compound, m.p. 117° , and a phenylisocyanate compound, m.p. 145° .

There is evidence of the presence of other undescribed substances among the hydrolysis products of the oat proteins.

The above work formed part of the general scheme of research on the proteins carried out under the auspices of the Adhesives Committee of the Department of Scientific and Industrial Research.

The Secretion of Pituitrin in Mammals, as shown by Perfusion of the Isolated Kidney of the Dog.

By E. B. VERNEY, Beit Memorial Research Fellow.

(Communicated by Prof. T. R. Elliott, F.R.S.—Received February 11, 1926.)

(From the Laboratories of the Medical Unit, University College Hospital Medical School, London.)

CONTENTS.

	PAGE
A. Experimental Method	490
B. Experimental Technique and Results	494
C. Histological Examination	509
D. Discussion	510
E. Summary and Conclusion	512
F. Protocols	513
References	516

INTRODUCTORY.

Organs of internal secretion may conveniently be considered as belonging to one of two groups. In the one the secretory product is continuously being poured into the circulation, the physiological condition which we are pleased to call normal being dependent on this occurrence for its very maintenance.

In the other group secretory activity is called into play only in conditions of stress when the normal physiological balance is upset to an abnormal degree, the extent of the activity being delimited by the return of the organism to its previous physiological condition.

There are three methods of experimental approach to the study of internal secretion, each of which by itself can furnish unequivocal evidence for the actual or potential secretion by a particular organ of some specific product into the blood stream during life. In the first case the arterial and venous bloods of the organ *in situ* or artificially perfused are subjected to chemical or biological assay: witness the secretion of sugar by the liver (1). In the second case the smallness of the organ or its inaccessibility, or both, preclude the possibility of dealing directly with its arterial and venous supply and return. Attention is therefore directed to analyses of the arterial and venous bloods of a larger volume of tissue containing the organ in question, supplemented by control analyses of the bloods after its operative removal: witness the secretion of adrenalin by the suprarenals (2). In the third case removal of the organ from the whole animal is undertaken, the signs and symptoms elicited by this means are noted, and the capability either of extracts of the organ under examination or of the gland substance to counteract these signs and symptoms, is tested: witness the secretion of thyroxin by the thyroid (3), and of insulin by the pancreas (4).

In the case of the pituitary body, physiologists have up to the present time limited their investigations on the function of this organ to the third method of experimental approach. This has recently met with success in some of the lower animals. In mammals, however, the results obtained after operative interference on the pituitary body and its neighbourhood have been varied and inconstant. We may correlate this fact with the intimate association which exists between the pituitary body and the base of the brain, making complete experimental removal impossible without extensive injury to the brain (Staderini (5), Tilney (6)). It is natural, therefore, that there should be disagreement as to the interpretation to be put upon the results of experimental removal, partial or "complete," some experimenters incriminating functional impairment of the pituitary body, others injury to the base of the brain as being responsible for the particular signs following the operation. Whichever view is taken, we must conclude that it has not been proven that the pituitary body plays an essential part in the production of these signs.

One sign which has sometimes been reported as following experiments on pituitary removal in mammals, and which recently has attracted considerable attention is polyuria. Vassale and Sacchi (7) reported it in some of their experiments and noted the low specific gravity of the urine. Pironne (8) on the other hand, states that the urine was normal in quantity in those dogs from which he had removed the pituitary body. Gemelli (9) observed it frequently during the first five to six days following "total" ablation. Paulesco (10) made no comment on the urinary output in his experimental animals. Crowe, Cushing and Homans (11) state that following total hypophysectomy in adult dogs the urinary output diminishes to anuria. In puppies, on the other hand, a transient polyuria was often observed and this was also frequently the case in those animals in which the pituitary stalk

was divided, and in those in which the anterior lobe was partially, or the posterior lobe completely, removed. Dean Lewis (12) also observed polyuria following injury to the hypophysis by the buccal route in 9 out of 18 dogs on which he experimented. In 1912, Frank (13) drew attention to the relations of the hypophysis to diabetes insipidus, and in the following year Von den Velden (14) showed that the polyuria of this disease could be completely controlled by subcutaneous injections of pituitrin.

The importance of the study of the polyuria sometimes resulting from operative interference on or in the neighbourhood of the pituitary body, lies in the possibility of its leading to a definite conclusion as to whether this polyuria, and presumably that of diabetes insipidus, is or is not due to a diminution in the hypothetical internal secretion of the pituitary body. At present both experimental and clinical evidence is notoriously equivocal on this subject. On the experimental side we have the evidence of Camus and Roussy (15) that the polyuria is due not to ablation of the hypophysis, but to a superficial lesion of the base of the brain in the optopeduncular space. This experience is acquiesced in by Houssay (16), Leschke (17) and Bailey and Bremer (18) as a result of their own experimental researches. Indeed, Camus and Roussy (*loc. cit.*) and Leschke (*loc. cit.*) are agreed that extirpation of the whole hypophysis (dogs—palatal route) does not produce polyuria if the brain base is avoided. On the clinical side Leschke (*loc. cit.*) has collected many cases in which destruction of the whole hypophysis by tumours, syphilitic and tuberculous disease, and cysticercus cellulosa, have not been accompanied by diabetes insipidus, whilst pathological processes involving the brain base in the optopeduncular region have been so accompanied. All these observers attribute the polyuria to a disturbance of the regulating function of the hypothalamus as regards the output of water and salts by the body. On the other hand, we have the presence in this region of a body from which an extract can easily be prepared which inhibits in the vast majority of cases the diuresis of diabetes insipidus whether clinical (Von den Velden (*loc. cit.*), Laison, Weir and Rowntree (19), Maranon (20), Blumgart (21)) or experimental (Bailey and Bremer (*loc. cit.*)). Camus and Roussy (22), however, state that the immediate polyuria following a lesion in the optopeduncular space in dogs is not controlled by pituitary extract, and that in the permanent experimental polyuria the results of opotherapy are instant, without lasting effect, and not specific.

It may be pointed out that a polyuria resulting from injury to, or disease in the hypothalamic region does not preclude the possibility of the hypophysis being concerned in its production. The only positive proof of its non-intervention would be the production of a polyuria as the result of an injury in this region subsequent to complete ablation of the pituitary body. Camus and Roussy (23) claim to have shown that this is so, but, as has already been pointed out, the anatomical disposition of the body absolutely precludes its complete removal without coincident injury to the brain. It may further be pointed out that the impotence of pituitrin to inhibit the polyuria in certain cases cannot be accepted as evidence against the hypophyseal etiology of this condition, unless the activity of the preparation is concurrently controlled by positive results on other cases. One must agree, however, with Bailey (24) that it is not yet proven that pituitary extract is the secretory product of the pars posterior, or indeed of any other part of the pituitary.

In 1925 Starling and Verney (25) published the results of their experiments on the secretion of urine as studied on the isolated mammalian kidney. They showed that when the dog's kidney was perfused by means of a heart-lung preparation, the urine gradually acquired the characters of that seen in experi-

mental and clinical diabetes insipidus, namely, a large output of water, low total tonicity, and low chloride percentage. The addition of pituitary extract to the circulating blood inhibited the polyuria in a dramatic fashion, and at the same time caused a marked rise in the percentage output of chloride and sometimes even in the absolute output. This effect invariably occurred, whilst every other means adopted to lower the urine flow, such as lowering the blood pressure or the addition of adrenalin, failed to bring about a concomitant rise in the urinary chlorides, a fall in blood pressure indeed causing the urinary chlorides to fall. The action of pituitrin was, therefore, regarded as being specific, and the suggestion was made that in the normal animal a pituitary principle or principles were being continually added to the blood by the tissues, and that the characters of the urine secreted by the isolated organ were due in large part to their absence.

A.—EXPERIMENTAL METHOD.

Experiments have, therefore, been performed with the object of substantiating or disproving this suggestion. The method adopted is in principle extremely simple, and falls into the second category of the methods enunciated in the beginning of this paper as affording unequivocal evidence of potential internal secretion.

A heart-lung-kidney is prepared and the effect on the quantity and composition of the urine of switching various parts of the tissue of another dog into perfusion-parallel with the kidney is observed. By the term perfusion-parallel in this paper is to be understood the diversion of some of the blood from the arterial side of the heart-lung to the particular tissue perfused, the efferent blood from this tissue being led back to the venous side of the heart-lung and so reaching eventually the isolated kidney, which is also connected with the arterial side of the heart-lung. The isolated kidney is, therefore, used as a test object by means of which the blood passing through it may be biologically assayed as to its content in pituitrin-like substances. Four series of experiments have been performed. In the first series the head and neck, with or without the right fore-limb, have been perfused in parallel with the kidney; in the second series the hind legs and pelvis; in the third the head and neck after exposure of the pituitary body, and in the fourth the head and neck subsequent to removal of the pituitary body.

The apparatus used is shown diagrammatically in fig. 1. A B C on the left-hand side represents the heart-lung preparation of Starling (26). From the arterial side of this preparation the blood passes to a Y-piece D. Some of

the blood flows by the lower limb of the Y through the warming coil E to a cannula F supplied with a thermometer and inserted into the renal artery of the kidney G. H represents a cannula placed in the ureter so as to allow the urine secreted to be collected for subsequent measurement and analysis. The venous blood from the kidney is collected *via* the graduated vessel L in three Dewar flasks 0, 1 and 2, and from these the blood is poured successively back to the venous reservoir A. The vessel L furnished below with a pinch-cock, allows the blood flow through the kidney to be accurately determined at any

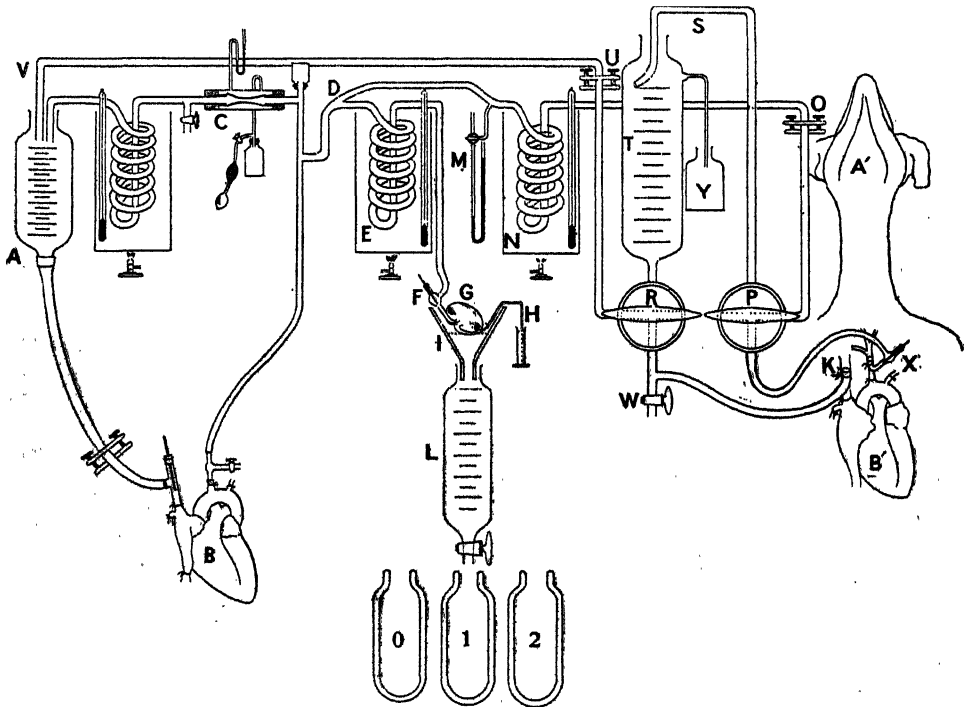


FIG. 1.

moment. The remainder of the arterial blood from the heart-lung passes by the upper limb of the Y-piece D *via* a warming coil N to a large bore three-way tap P. When P and R are turned through 180° from the position shown in the figures, the blood passes *via* the tube S to a graduated reservoir T, from which it flows *via* the tap R and tube U V back to the venous reservoir of the heart-lung. A screw clip O regulates the quantity of blood shunted through T and at the same time affords a rapid means of adjustment of the pressure of the blood supply to the kidney, which is measured by a Hg. manometer at M. A screw clip U controls the quantity of residual blood in T: Y is a vessel so

placed that it can collect any overflow of blood from T should this vessel become overfilled from maladjustment of the screw clips O and U. A' represents the ventral surface of a dog's head, and the heart of the animal is shown at B'. The cannula X supplied with a thermometer is shown pointing distally from the heart and tied into the brachiocephalic trunk. It is connected with the third limb of the tap P. The cannula K is inserted into the superior vena cava and communicates in similar fashion with the third limb of the tap R. The clip W affords a means by which the venous blood from the head may be collected in the period during which the head is being washed through with arterial blood, preparatory to the blood being allowed to flow back to the heart-lung reservoir. The shunt O P S T R U V is for the purpose of maintaining a blood flow through it approximately equivalent to what experience has taught as to the flow to be expected through the head A'. It further obviates any gross changes of pressure and temperature at the renal cannula when the head is switched into, and later out of the circuit. Consequently when the cannula X and K are in position the head may be substituted for the shunt by simply turning the tap P

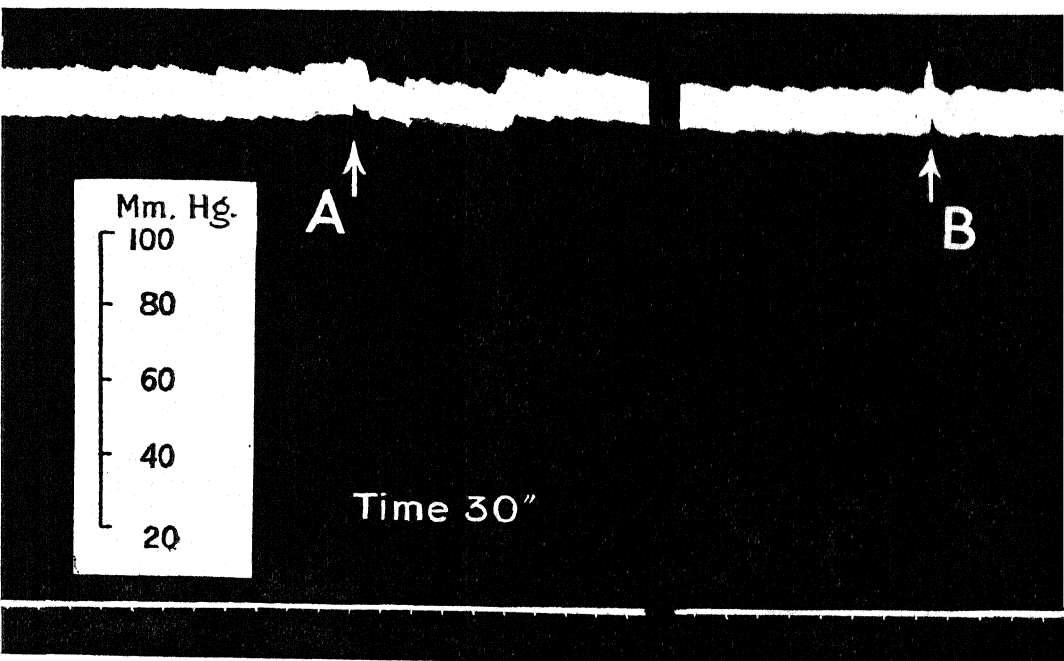


FIG. 2.—Blood pressure at renal arterial cannula. At A the legs and pelvis were switched into parallel with the kidney and at B were switched out of the circuit. Tracing from the experiment recorded in fig. 7.

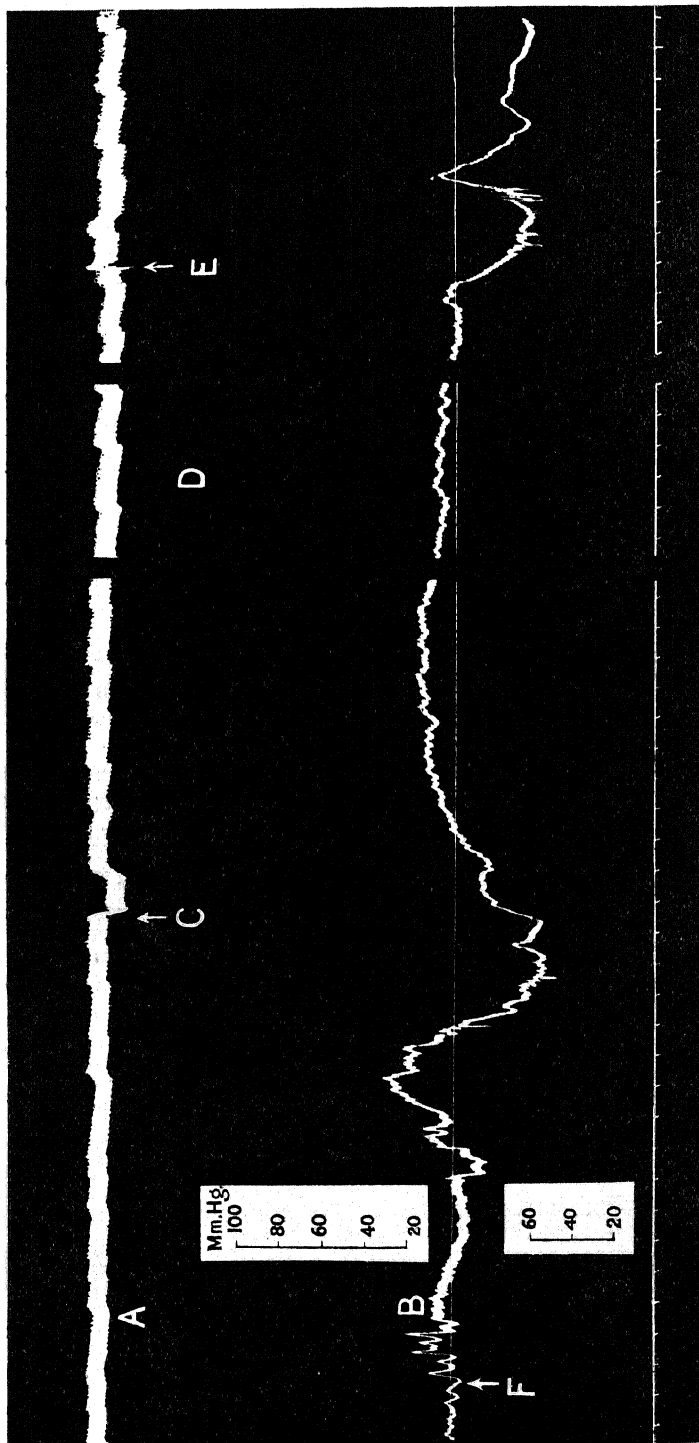


FIG. 3.—A.—b.p. at renal cannula. B.—Femoral b.p. of dog A'B' on right of fig. 1. At F 170 mgms. heparin given intravenously. At C the head and neck were switched into perfusion-parallel with the kidney, and at E were switched out of the circuit. D.—Sample part of b.p. trace in the interval between C and E. Time marking = 30 seconds. This record is taken from the experiment illustrated in fig. 8 (*q.v.*).

through 180°, the tap W being open. Any small change of pressure at the kidney is then rapidly overcome by manipulating the clip O, and in this way the necessity of altering the arterial resistance C or the venous inflow of the heart-lung preparation is avoided. The extremely small nature of the disturbance of the renal blood pressure when the legs or head of the animal are switched into and out of perfusion-parallel with the kidney, is demonstrated in figs. 2 and 3 respectively.

The first 400 to 500 c.c. of blood perfused through the head are collected at W and discarded. By appropriately turning the tap R the superior vena caval cannula K is connected directly *via* U and V with the venous reservoir of the heart-lung, and the effect on the kidney of shunting part of the blood through the head is noted. After an appropriate interval the taps P and R are returned to their original position, and the urine collected over a further period under these altered conditions.

The animal A'B', the taps R and P, and the top of the venous reservoir A are on the same level, the heart-lung table being 25 cm. below the table on which the dog A'B' is placed.

In those experiments in which the lower limbs and pelvis have been switched into perfusion-parallel with the kidney, the identical apparatus is used, the cannula X being inserted distally into the aorta just above its bifurcation and K similarly placed alongside of it in the inferior vena cava. In all the experiments the heart-lung was ventilated with air. The renal blood pressure was registered on a kymograph, as was also the blood pressure in the femoral artery of the animal A'B'. The hæmoglobin concentration of the blood was estimated as HbCO colorimetrically. The urinary and serum chlorides were determined by the method of Millard Smith (27).

In the figures of this paper where the urine flow is charted, the time at which the flow is recorded is the mean time of the interval through which the particular sample of urine is collected. In the same way the blood-pressure figure is the mean pressure for the corresponding interval, and is obtained from measurement of the pressure trace at the end of the experiment.

B.—EXPERIMENTAL TECHNIQUE AND RESULTS.

Series I.—The effect on the urinary secretion of the isolated kidney of switching the head-neck into perfusion-parallel with it.

Five dogs were, as a rule, used for each experiment. Two were bled to death under morphia (1 mgm. morphine tartrate per kilo.) and C. and E. anæsthesia, and the blood defibrinated. A third animal was then taken, a preliminary

dose of morphia and C. and E. given and chloralose injected intravenously in a dose of 0.1 gm. per kilo. body weight.

A tracheal cannula was inserted, artificial respiration established, the chest opened, the phrenic nerves excised, and the left subclavian and brachiocephalic arteries and the superior caval and azygos veins isolated and loose ligatures passed around them. The blood pressure was recorded from a cannula in the right femoral artery, and the animal then defibrinated by bleeding from the left femoral artery and allowing defibrinated blood to flow *via* a warming coil into the external jugular vein at such a rate that the animal's blood pressure was maintained fairly constant. Defibrination was not always pushed to completion, incoagulability of the total blood being sometimes attained by injecting 100 to 150 mgms. of heparin intravenously after a preliminary lavage of the animal with a litre of defibrinated blood.

A fourth dog was then anaesthetised with chloralose after a preliminary dose of morphia and C. and E., bled to the extent of 300 c.c., and 350 c.c. warm normal saline injected intravenously. A cannula was placed in the left ureter and the left renal artery exposed, a ligature being inserted loosely around it.

A fifth dog was used in the preparation of the heart-lung, the kidney of the fourth dog excised and perfused as described in the paper by Starling and Verney (*loc. cit.*). A little urea was often added to hasten the urine flow. When the polyuria was well established, the cannulae X and K were inserted and the left subclavian artery and azygos vein ligatured, as was done by Starling and Anrep (28) in their experiments on the central and reflex regulation of the circulation.

When approximately 500 c.c. of blood had been washed through the head, the tap R was turned and W closed so that the blood from the head now ran back to the heart-lung. In fig. 4 the results of one experiment of this series are charted. About 2,500 c.c. of blood were circulating at the beginning of the experiment, and 2 gms. of urea were added to the blood.

The initial polyuria and fall in the urinary chloride are well shown. At G the head and neck were switched into perfusion-parallel with the kidney, 500 c.c. of blood being washed through the head and discarded before allowing its return to the venous reservoir of the heart-lung. The efficacy of washing was shown by there being no abnormal rise in the Hb per cent. of the circulating blood as a result of switching-in the head, although the relative Hb concentration of the blood of the head-dog was 133 per cent. It is clear from the figure that switching-in the head caused a marked and ingravescient fall in the output of water and a less marked rise in the urinary chloride percentage. The flow through

the head was 215 c.c. per minute, the temperature and pressure of blood supply being 38° and 88 mm. Hg respectively. At H the head was switched out of the circuit, and there commenced, after a latent period of 25 minutes, a renewed polyuria accompanied by a progressive fall in the urinary chloride. The blood

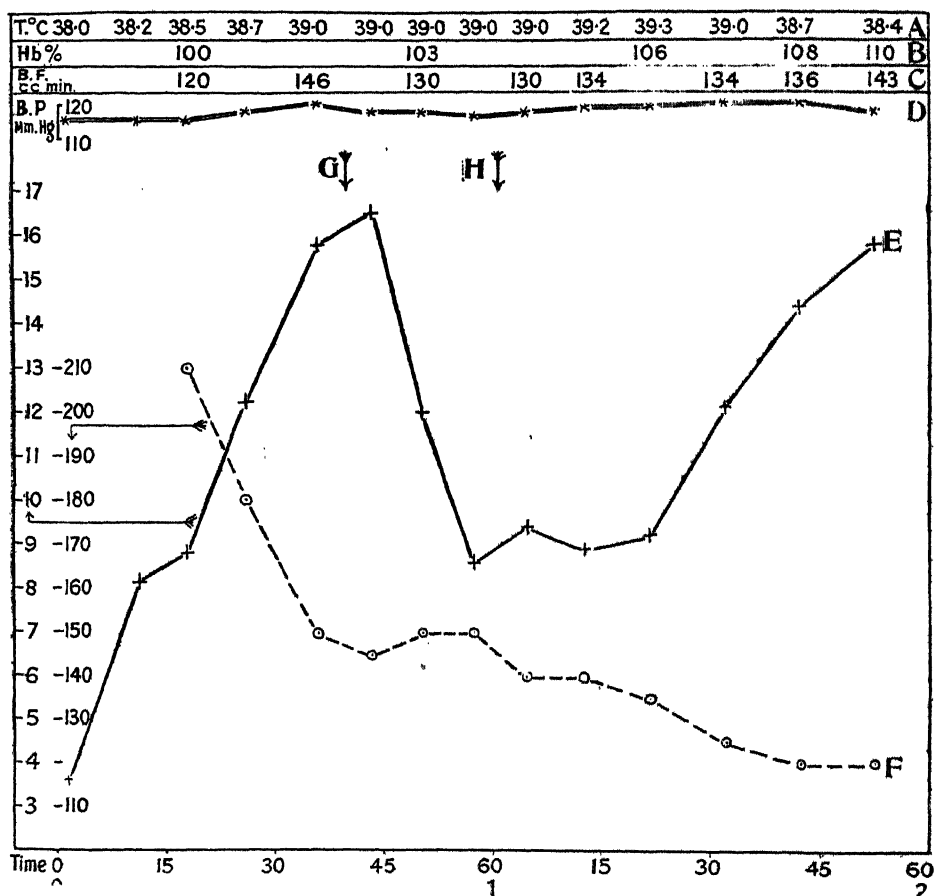


FIG. 4.—A = Temp. of blood at renal cannula. B = Hb per cent. of blood, relative to the initial sample. C = Renal blood flow in c.c. per min. D = B.P. in mm. Hg in renal artery. At "G" head, neck and right fore-limb were switched into perfusion parallel with the kidney, and at "H" were switched out of the circuit. E = Urine flow in c.c. per 15 min. F = Urinary Chloride (as NaCl) in mgms. per cent.

was well oxygenated throughout the experiment, and no pulmonary cedema supervened. The kidney weighed 44 gms. and showed no abnormality on macroscopic examination. The urine samples were clear and contained no protein excepting a minute trace which was present in the first and second samples. It is further to be noted that there was a small diminution in blood

flow through the kidney (from 146 to 130 c.c./min.) when the head was switched into the circuit, and that this gradually increased again up to 143 c.c./min. when the head was switched off.

Fig. 5 illustrates the results of a similar experiment. About 2,500 c.c. of

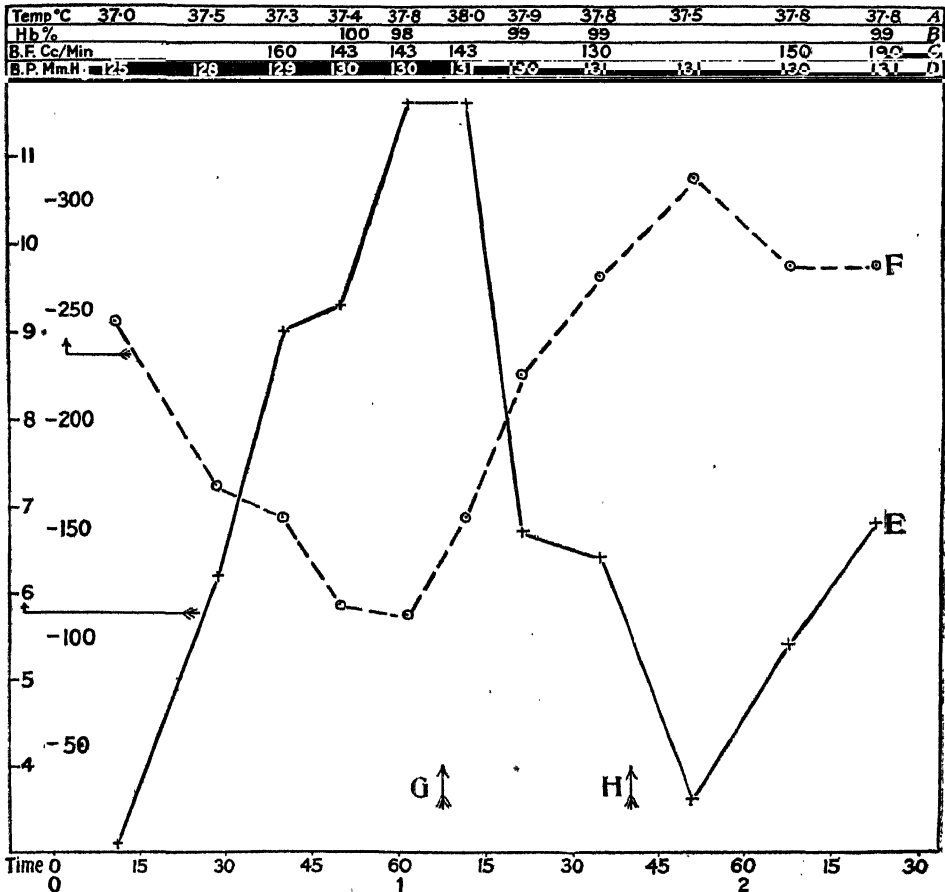


FIG. 5.—A = Temp. of blood at renal cannula. B = Hb per cent. of blood, relative to the initial sample. C = Renal blood flow in c.c. per min. D = B.P. in renal artery in mm. Hg. At "G" head, neck and right fore-limb were switched into perfusion parallel with the kidney, and at "H" were switched out of the circuit. E = Urine flow in c.c. per 15 min. F = Urinary Chloride (as NaCl) in mgms. per cent.

blood were circulating at the beginning of the experiment and 3 gms. of urea were well mixed with this.

The head, neck and right fore-limb were washed through to the extent of 750 c.c. before allowing the blood issuing from the superior vena cava to return to the venous reservoir. The Hb percentage of the blood of the head-dog

just before switching over was 121 per cent., that of the 750 c.c. of washings was 102 per cent., and from the figure it will be seen that the return of the venous blood from the superior vena cava was unaccompanied by any change in the percentage of Hb in the circulating blood of the preparation. The blood flow through the head, neck and right fore-limb was 263 c.c./min. and the temperature and pressure of the blood supplied *via* the brachiocephalic artery were 38° C. and 100 mm. Hg respectively. It will be seen that switching the head into the circuit produced a marked and ingravescient fall in the urinary flow, and an equally well-marked and ingravescient rise in the percentage of chloride in the urine. In this experiment indeed there was actually a rise in the absolute amount of chloride excreted during the period G to H, compared to that excreted immediately before switching the head into the circuit. At H the head and neck were switched out of the circuit, the blood now passing back *via* the shunt S T (fig. 1) to the venous reservoir. After a short latent period it is seen that the polyuria returned and the urinary chloride began to fall again. Further, it is to be noted that a small diminution in renal blood flow again resulted from the passage of the blood through the head of the animal—143 to 130 c.c./min.—and that this recovered to 150 c.c./min. during the second polyuria, and reached a figure of 190 c.c./min. by the end of the experiment. The blood pressure at the renal cannula was constant throughout. The kidney weighed 50 gms. and macroscopically appeared somewhat oedematous. The blood was well oxygenated throughout the experiment and no pulmonary oedema occurred. The merest trace of Hb was present in all the urine samples.

A further experiment of this series is summarised in Protocol 1.

These experiments prove conclusively that the blood of the heart-lung-kidney preparation, when perfused through the head-neck and right fore-limb of another animal, picks up some substance which reacts on the kidney in a manner qualitatively identical to that of pituitrin.

Series II.—The effect on the urinary secretion of the isolated kidney of switching the pelvis and lower limbs into perfusion-parallel with it.

The method adopted in this series was exactly analogous to that used in the first series of experiments. The chest of the animal, however, was not opened. The left carotid artery was exposed and a tracheal cannula inserted. Respiration was natural. After opening the abdomen the pelvic colon was divided between ligatures, and the lowest parts of the abdominal aorta and the inferior vena cava exposed. All neighbouring branches and tributaries of these vessels were then ligatured. The animal was defibrinated *via* the external jugular

vein and left carotid artery, and double ligatures placed loosely around the aorta and inferior vena cava just above their divisions. A mercury valve adjusted to a pressure of 102 mm. Hg was connected with a cannula in the left carotid artery pointing towards the heart. This valve was brought into operation just before switching the legs into perfusion-parallel with the kidney, so that the animal's blood pressure was automatically not allowed to rise above 102 mm. Hg.

The results of one experiment are charted in fig. 6. At H the lower legs and pelvis were switched into the circuit by inserting the cannulae H and K (fig. 1) into the aorta and inferior vena cava respectively. 350 c.c. of blood were washed

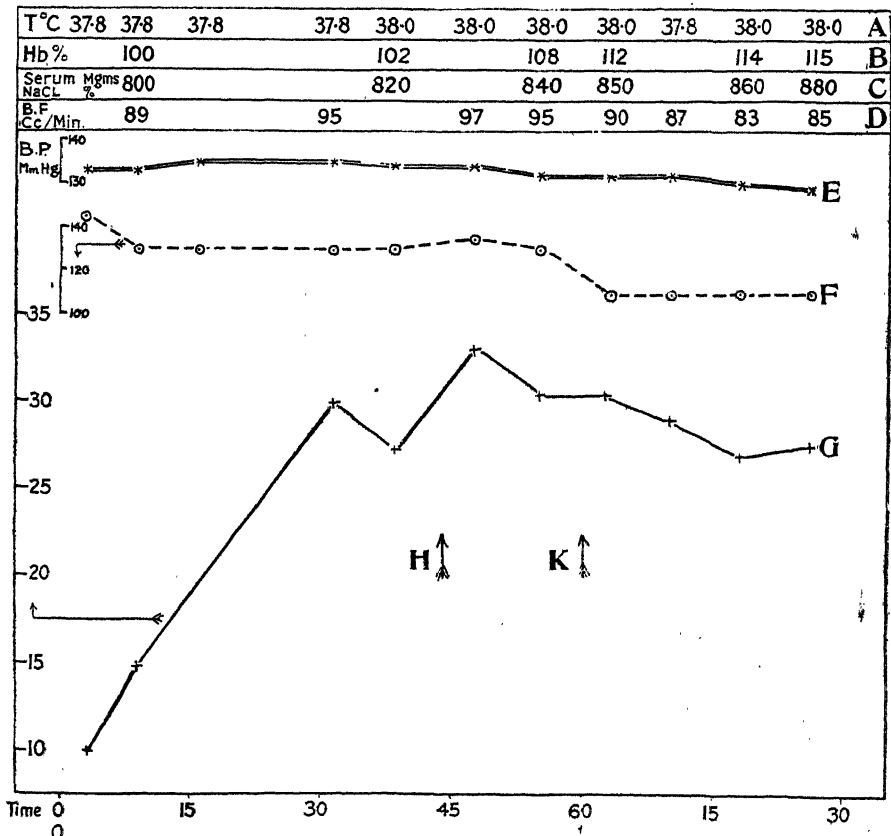


FIG. 6.—A = Temp. of blood at renal cannula. B = Hb per cent. of blood, relative to the initial sample. C = Serum Chloride (as NaCl) in mgms. per cent. D = Renal blood flow in c.c. per min. E = B.P. in renal artery in mm. Hg. At "H" hind legs and pelvis were switched into perfusion parallel with the kidney, and at "K" were switched out of the circuit. F = Urinary Chloride (as NaCl) in mgms per cent. G = Urine flow in c.c. per 15 min.

through before allowing the return blood from the inferior vena cava to pass back to the venous reservoir of the heart-lung. At K the legs and pelvis were switched out of the circulation. It will be observed that the passage of the blood through the legs of the animal had no appreciable effect on the rate of urine flow from the isolated kidney or on its chloride content. The blood flow through the legs and pelvis at time 0.50' was 94 c.c./min. and at 0.58' 91 c.c./min. The temperature and pressure of the blood supply to the legs was 38° C. and 113 mm. Hg respectively. The mercury valve was inserted in the carotid in order to maintain the blood pressure of the non-perfused part of the animal at a figure below that of the pressure of supply to the legs. Any risk of blood passing by small anastomotic channels from the top of the animal to the perfused part and so eventually reaching the kidney was in this way excluded. It will be seen that there was no diminution in blood flow through the kidney as a result of switching the legs into the circuit. The kidney weighed 36 gms. and was normal on macroscopical section. The blood was well oxygenated throughout and no pulmonary oedema supervened. All the urine samples were clear and contained no protein.

Objection might be taken to this experiment on the score that the blood flow through the legs and pelvis was much smaller than that through the head and neck in the first series of experiments. Although there seemed to be no *a priori* reason to suppose that the amount of pituitrin-like substance picked up by the blood during its passage through the tissues should be less the smaller the blood flow, it was thought worth while to control the negative result obtained by other experiments in which the femoral and sciatic nerves were divided. Fig. 7 is the record of such an experiment. The technique was the same as previously described with the addition that the sciatic and femoral nerves were exposed in the animal to be perfused.

About 2,500 c.c. blood were circulating at the beginning of the experiment, and the urine started to flow immediately the artificial circulation was established through the kidney. 1.5 gms. urea were then added to the blood. Immediately before switching in the legs the sciatic and femoral nerves were divided; 550 c.c. blood were washed through the legs before allowing the return blood to run back to the venous reservoir. The mercury valve in the carotid was adjusted at 95 mm. Hg, and the blood pressure at the cannula in the aorta was 100 mm. Hg. The blood flow through the legs and pelvis, taken 10 minutes after switching them into parallel with the kidney, was 250 cc./min. and the temperature of supply 36° C. It will be seen from the figures that if any effect at all was produced on the urine flow by the passage of the

blood through the legs and pelvis, it was in the direction of increasing the rate of rise. Further, the fall in the percentage of chlorides in the urine was not inhibited. At B, the legs and pelvis were switched out of the circuit. That the kidney was responsive to pituitrin was shown by adding 0.03 c.c. "Infundin"

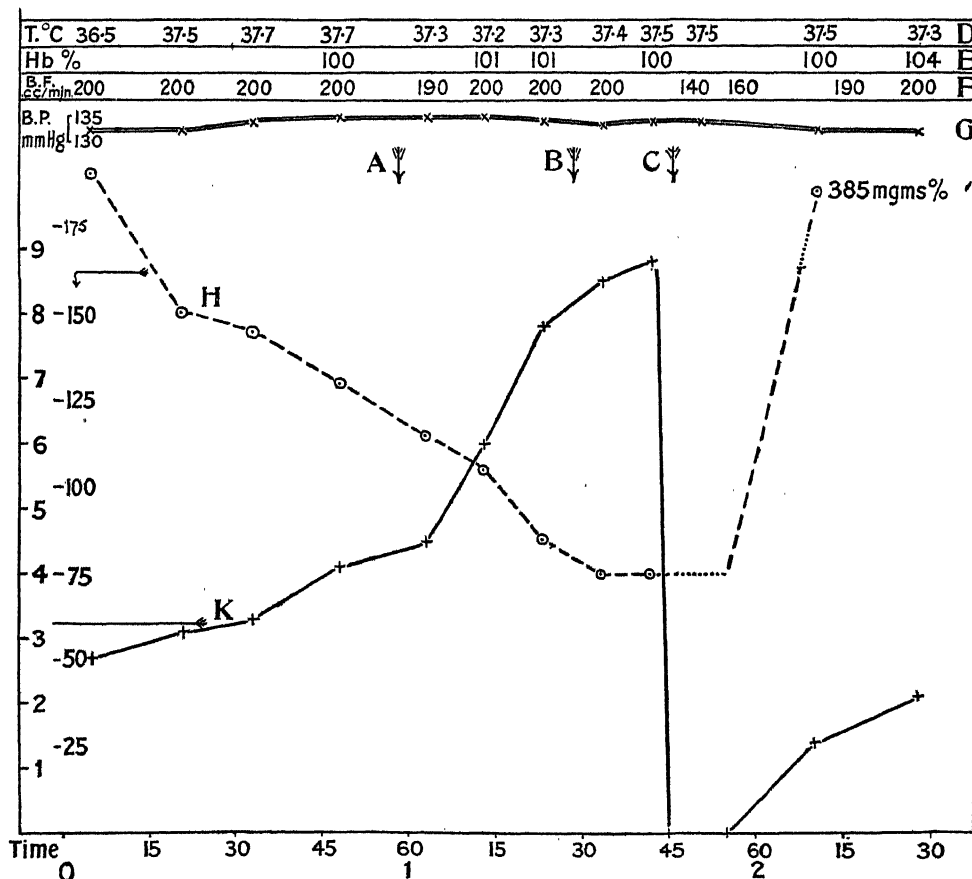


FIG. 7.—Temp. of blood at renal cannula. E = Hb per cent. of blood, relative to the initial sample. F = Renal blood flow in c.c. per minute. G = B.P. in mm. Hg. At "A" hind-legs and pelvis were switched into perfusion-parallel with the kidney, and at "B" were switched out of the circuit. At "C" 0.03 c.c. "Infundin" added. H = Urinary chloride (as NaCl) in mgms. per cent. K = Urine flow in c.c. per 15 Min.

to the circulating blood. The urine flow was temporarily completely inhibited, and on its return the large percentage rise in the urinary chloride was made manifest. No pulmonary oedema was encountered, and the blood was well oxygenated throughout the experiment. It will further be observed that the blood flow through the kidney suffered no permanent inhibition when the

legs were switched into the circuit, and that there was no change in the Hb concentration of the blood during this interval as compared to the preceding one. The kidney weighed 44 gms. and showed no abnormality on section. Two further experiments in this series are recorded in Protocols 2 and 3.

The conclusion is now justified that no pituitrin-like principles are contributed by the tissues to the blood artificially perfused through the lower limbs and pelvis of a dog. It remains to attempt to locate the particular tissue in the head-neck and right fore-limb which is responsible for this contribution. Naturally, the pituitary body has the first claim on our experimental investigation.

Series III.—The effect on the urinary secretion of the isolated kidney of switching the head and neck, after exposure of the pituitary body, into perfusion-parallel with the kidney.

The technique in this series of experiments was exactly analogous to that in Series I. It differed merely in that the pituitary body of the animal, the head of which was to be perfused, was exposed. The route chosen for exposure was the temporal one and the method adopted differed little from that practised by Paulesco (*loc. cit.*) and by Cushing (29). The left temporal muscle was stripped from its origin, the superficial temporal vessels ligatured and the belly of the muscle removed by carrying an incision through it at the level of the zygoma. Two to two and a half centimetres of the zygomatic arch were removed, all hæmorrhage being arrested by sealing the medullary vessels with Horsley's wax. The jaw was widely opened, and the whole temporal fossa then came into view. The skull was trephined, and the edges of the hole freely cut away in all directions so that an opening about 4 cm. in diameter was obtained. All hæmorrhage was again arrested, the dura punctured in the centre of the opening, and radial incisions made in it towards the periphery. The dural sectors were in turn reflected on to the surface of the skull, the temporal lobe gently lifted with a retractor, when a clear view of the pituitary body was readily obtained. The cerebro-spinal fluid was absorbed by numerous pledglets of wool, a small wool drain placed between the pituitary region and the exterior, and the field of operation covered with a warm salined woollen pad. It was found unnecessary in these acute experiments to decompress the contra-lateral side of the brain as well, since the large area of bone which could be cut away after the belly of the temporal muscle had been removed, allowed the brain to be raised to the small extent required to expose the pituitary, without causing any undue compression. It will be shown in a later paper that exposure of the pituitary in this manner has no effect either on the rate of urine flow in the dog

operated upon or on the percentage of urinary chloride. Table I records a typical experiment of this series. On switching to the head, 400 c.c. were washed through before allowing the return blood to flow back to the heart-lung.

Table I.

Dogs 1 and 2 bled under C. and E.

Dog 3, 10.2 kilos. C. and E. Chloralose. *Pituitary exposed.* Chest opened and vessels prepared for head perfusion. Right subclavian artery ligatured distal to right vertebral.

Dog 4, 10.5 kilos. Morphia. C. and E. Chloralose. Bled 300 c.c., 350 c.c. normal saline i.v. Left renal artery cleaned and ureteric cannula inserted.

Dog 3 defibrinated almost completely and given 150 mgms. heparin i.v.

Dog 5, 9.7 kilos. Morphia. C. and E. Chloralose. Heart-lung prepared. Heart-lung-kidney completed. 3 gms. urea added.

Time.	T° C. Kidney.	B.P. Mm. Hg.	B.F. c.c./min.	Hb. Per cent.	Serum Solids per cent.	Serum. NaCl. Per cent.	Urine. c.c./15 min.	Urine. NaCl. Per cent.
5.01—5.17	37.0	136	107	—	—	—	3.0	120
5.17—5.33	37.1	136	107	—	—	—	3.5	135
5.33—5.42	37.3	132	—	—	—	—	3.7	125
5.42—5.55	37.4	140	—	100	—	—	5.4	105
5.55—6.05	37.6	144	122	—	—	—	7.9	85
6.05—6.19	37.4	142	—	98	7.42	810	6.0	85
6.19—6.30	37.0	144	113	101	7.56	810	4.0	100 ← A.
6.30—6.52	37.0	144	100	99	7.46	810	2.1	205
6.52—7.14	37.0	145	107	101	7.73	810	1.8	245

At A head and neck of dog 3 switched into perfusion-parallel with the kidney.

The blood flow through the head was 188 c.c./min. and the pressure of supply 106 mm. Hg. The resultant inhibition of urine flow and concomitant percentage rise in chloride, unaccompanied by any change in the blood pressure, are well shown. The diminution in blood flow from 122 to 100 c.c. per minute is also to be noted. The blood was well oxygenated throughout, and the urine samples were clear and contained no Hb nor protein. The kidney was normal on macroscopical examination.

Another experiment giving qualitatively identical results is recorded in Table II. The diminution in blood flow resulting from switching the head into the circuit was, however, in this experiment minimal or non-existent.

Table II.

Dogs 1 and 2 bled under C. and E.

Dog 3, 12 kilos. C. and E. Chloralose 1.1 gm. *Pituitary exposed*. Chest opened and vessels prepared for head perfusion.

Dog 4, 11 kilos. Morphia. C. and E. Chloralose 1.1 gm. Bled 350 c.c., 400 c.c. normal saline .v. Left renal artery cleaned and ureteric cannula inserted.

Dog 5, 12 kilos. Morphia. C. and E. Chloralose. Heart-lung prepared.

Dog 3 defibrinated. Heart-lung-kidney completed. 3 gms. urea added.

Time.	T °C. Kidney.	B.P. Mm. Hg.	B.F. c.c./min. Kidney.	Hb. Per cent.	Urine. c.c./15 min.	NaCl. Mgms. Per cent.
5.03—5.09	36.0	126	—	—	8.5	40
5.09—5.17	36.8	133	182	—	8.3	40
5.17—5.27	36.3	132	194	—	7.8	44
5.27—5.37	35.9	132	200	—	7.7	35
5.37—5.48	35.9	126	—	—	7.7	35
5.48—5.58	35.9	126	200	100	7.5	55 ← A.
5.58—6.08	35.7	125	194	101	4.6	105
6.08—6.18	36.0	122	—	—	3.7	145

At A head, neck and right fore limb of dog 3 switched into perfusion-parallel with the kidney.

The urine samples were all clear and contained no Hb nor protein. The kidney was normal on macroscopic section. Table III records a further experiment of this series, and the results are seen to be qualitatively identical to those obtained in the other experiments of this series and of Series I.

A further point of interest arose, however, in this experiment. The first sample of urine contained a little blood but no free hæmoglobin. The second contained both red blood corpuscles and Hb, and the third Hb only. This persisted up to the time during which the tenth urine sample was collected, viz., 4.55 to 5.02, and in this sample red blood cells re-appeared. The last two specimens contained red blood cells only. The increase in the degree of hæmolysis before and the decrease after switching the head into the circuit are paralleled by the fall and rise respectively in the chloride concentrations of the urine. Starling and Verney (*loc. cit.*) have already commented on this phenomenon which resulted in their experiments from the addition of pituitrin to the blood of the heart-lung-kidney preparation.

Two further experiments of this series are recorded in Protocols 4 and 5.

So far, then, we must conclude that exposure of the pituitary body does not

Table III.

Dog 1 bled under C. and E.

Dog 2. 8.5 kilos. C. and E. Chloralose 0.9 gm. *Pituitary exposed*. Chest opened and vessels prepared for head perfusion.

Dog 3. Morphia. C. and E. Chloralose. Bled 300 c.c., 400 c.c. normal saline i.v. Left renal artery cleaned and ureteric cannula inserted.

Dog 4. Morphia. C. and E. Chloralose. Heart-lung.

Heart-lung-kidney completed. 5 gms. urea added.

Time.	T° C. Kidney.	B.P. Mm. Hg. Kidney.	B.F. c.c./min. Kidney.	Hb. Per cent.	Urine. c.c./15 min.	NaCl Mgms. Per cent.
3.48—3.54	35.0	147	—	—	7.7	535
3.54—4.01	35.0	144	—	—	8.3	368
4.01—	35.0	148	—	—	—	290
4.16—4.22	38.0	146	170	—	14.8	320
4.22—4.29	37.8	144	190	—	20.6	230
4.29—4.34	37.3	144	180	100	24.6	230
4.34—4.38	—	145	—	—	23.9	235
4.48—4.53	38.0	139	—	—	20.1	230 ← A.
4.53—4.58	38.0	139	170	—	18.0	290
4.58—5.02	38.0	146	—	104	17.6	410
5.02—5.07	38.0	146	—	—	12.3	490
5.07—5.15	37.8	144	—	—	8.5	515

At A head, neck and right fore limb of dog 2 switched into perfusion-parallel with the kidney.

influence the donation of a pituitrin-like principle by the tissues of the head and neck to blood passing through them. It remains to determine whether this effect is still obtainable after the pituitary body has been removed.

Series IV.—The effect on the urinary secretion of the isolated kidney, of switching the head and neck, after removal of the pituitary body, into perfusion-parallel with the kidney.

The technique in this series was exactly analogous to that in Series III. The pituitary body was exposed in the animal, the head of which was later to be perfused. The third nerve on the left side was divided as it passed from its external cerebral origin to the point where it traversed the dura to gain the region of the cavernous sinus. After collecting from cannulae in the ureters

sufficient urine for analysis, the left temporal lobe was again gently elevated, the pituitary body freed from its connection to the base of the pituitary fossa and removed with long forceps. Occasionally the body—anterior and posterior lobes—came away cleanly in one piece and the removal was, as far as could be determined with the naked eye, complete. More frequently, however, the body was removed in fragments. Hæmorrhage was very slight, a fact which is doubtless to be correlated with the rapid and profuse branching into vessels of almost capillary calibre of the sheaf of vessels streaming down from the circle of Willis around the stalk of the hypophysis (Dandy and Goetsch (30)). The operations both of exposure and removal were performed under direct illumination from a frontal lamp, so that stereoscopic vision was not interfered with. Table IV records the results of an experiment of this series. It will be seen that removal of the pituitary is followed after a latent period of an hour by an ingravescently profuse polyuria, and by a concomitant fall in the percentage of chloride in the urine. These acute effects of pituitary removal will be considered more fully in a later paper. At present it suffices to note their occurrence and to state that the resultant polyuria and low chloride percentage respond to intravenous injections of pituitrin in a manner exactly analogous to that of the polyuria and low urinary chloride of the isolated kidney. After the polyuria was established the chest was opened and the animal prepared for head perfusion. The remaining steps in the experiment were exactly as have already been

Table IV.

Dog 1, 11 kilos. Morphia. C. and E. Chloralose. Pituitary exposed. Ureters cannulated extraperitoneally. B.P. from right femoral artery.

Time.	Rectal T° C.	B.P. Mm. Hg.	Urine, Right and Left. c.c./15 min.	Urine. NaCl Mgms. Per cent.
11.30—12.10	37.1	116	0.9	490
Remove Pituitary.				
12.20— 1.15	37.5	116	0.8	175
1.15— 1.40	37.5	122	2.1	125
1.40— 1.58	37.5	122	2.8	115
1.58— 2.11	38.0	122	3.8	110
2.11— 2.23	38.5	122	5.5	90

Chest opened and vessels prepared for head perfusion. Defibrinated.

Table IV—continued.

Dogs 2 and 3 bled under C. and E.

Dog 4. Morphia. C. and E. Chloralose. Bled 500 c.c., 550 c.c. normal saline i.v. Left kidney prepared.

Dog 5, 11 kilos. Morphia. C. and E. Chloralose. Heart-lung.

Dog 6. Morphia. C. and E. Bled. Heart-lung-kidney prepared. 2 gms. urea added.

Time.	T° C. Kidney.	B.P. Mm. Hg.	B F. c.c./min.	Hb. Per cent.	Urine. c.c./15 min.	Urine. NaCl Mgms. Per cent.
5.40—5.52	38.2	129	—	—	2.9	435
5.52—6.00	38.3	128	188	—	4.6	350
6.00—6.07	38.1	129	—	100	5.0	335
6.07—6.20	37.8	128	200	—	6.3	290
6.20—6.29	37.4	126	—	—	5.6	345 ← A.
6.29—6.36	37.4	126	230	105	6.9	300
6.36—6.43	37.9	127	—	—	6.9	260
6.43—6.50	37.8	128	230	110	8.1	255
6.51—7.00	37.9	132	—	—	10.7	250 ← B.
7.00—7.10	38.0	131	—	—	9.0	250

At A, head, neck and right fore-limb of dog 1 switched into, and at B out of, perfusion-parallel with the kidney.

described. About 2,500 c.c. of blood were circulating. The urine flow began immediately the artificial circulation was established and before the urea (2 gms.) was added. It will be seen from the table that on switching into the circuit the head and neck of the polyuric animal at A, there resulted merely a transient and minimal fall in the water and rise in the chloride outputs, and that this effect rapidly disappeared. The rate of water output then gradually increased and the chloride percentage continued its progressive fall while the blood was still being shunted through the head. 500 c.c. of blood were washed through the head and discarded before allowing the return to the heart-lung. The blood flow through the head was 300 c.c./min. and the temperature and pressure of supply 37.5° C. and 95 mm. Hg respectively. It is to be noted that there occurred an actual increase in the rate of blood flow through the kidney during the time that the head and neck were in the circuit as compared to the rate immediately before, and this in spite of a small fall in blood pressure from 128 to 126 mm. Hg. The blood was well oxygenated throughout. The kidney weighed 77 gms.

The results of another experiment in this series are recorded in fig. 8. The operative procedure was identical to that described in the last experiment. The increased rate of urine flow in the whole dog was obvious to the eye 55 minutes after pituitary removal. After the animal had been prepared for head and neck perfusion it was defibrinated to the extent of 500 c.c. and 170 mgms. of heparin injected intravenously. When the heart-lung-kidney preparation had been made, the urine started to flow immediately the artificial circulation was established. Three gms. of urea were then added to the blood.

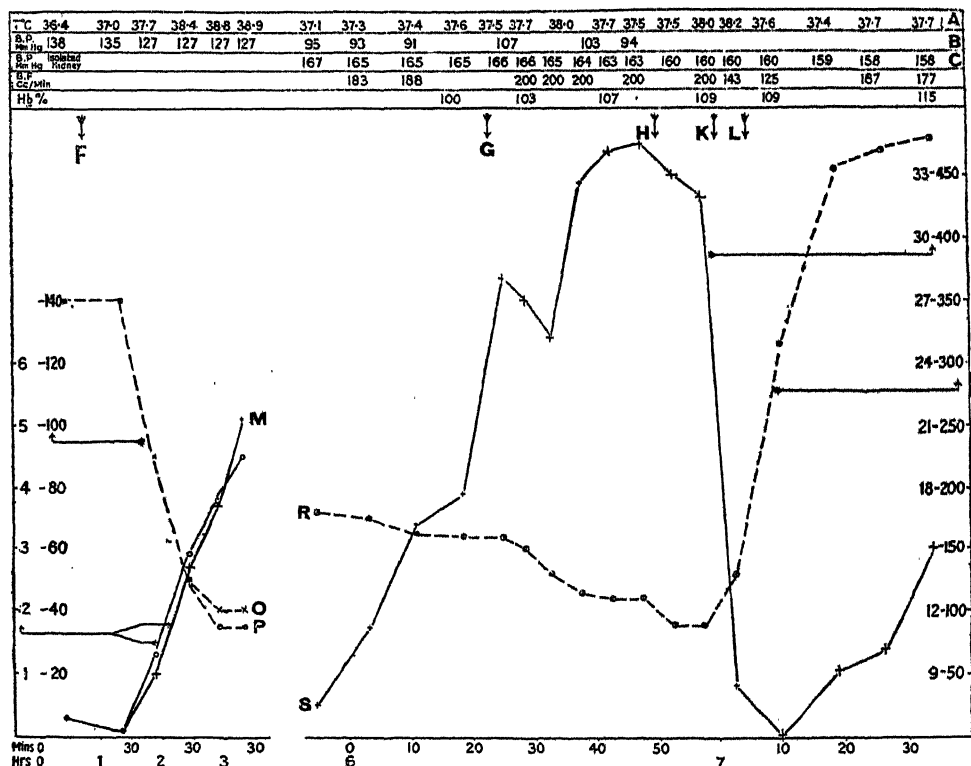


FIG. 8.—A = T °C. of intact Dog (rectal), and of blood in renal arterial cannula of isolated kidney. B = B.P. mm. Hg in femoral artery of intact dog. C = B.P. mm. Hg in renal artery of isolated kidney. D = Blood Flow in c.c./min. E = Hb per cent. relative to the initial blood sample. M = Urine Flow of Rt. kidney. N = that of Left in c.c./15 min. O = Urinary Chloride (as NaCl) in mgms. per cent. Rt. kidney, and P = that of Left. R = Urinary Chloride (as NaCl) in mgms. per cent., isolated kidney. S = Urine Flow isolated kidney in c.c./15 Min. At F the Pituitary Body was removed from the intact dog. At G the head, neck and right fore-limb were switched into perfusion parallel with the isolated kidney, and at H were switched out of the circuit. At K 0.02 c.c. "Infundin" was added to the blood perfusing the isolated kidney, and at L a further 0.01 c.c. was added.

At G the head and neck of the polyuric animal were switched into parallel with the kidney. There was a small and quite transient fall in the urine flow which then rapidly mounted again to the large value of 34.5 cc./15 min. The urinary chloride showed no appreciable break in its rate of fall as the result of the head shunt. Further, the blood flow through the kidney was actually greater during this period than at a time immediately preceding the operation of switching in the head and neck. The blood flow through the head, neck and right fore-limb was 188 c.c./min., the temperature of supply 37° C. at 6.25, and at 6.41 177 c.c./min. and 37° C. respectively. That the kidney was responsive to pituitrin was shown by the marked effect which its addition to the blood exerted on the rate of flow of the urine and on its percentage content in chloride. The blood was well oxygenated throughout. The kidney weighed 36 gms. and was normal macroscopically.

Another experiment of this series is recorded in Protocol 6.

The conclusion may justifiably be drawn, therefore, from this series that the operation of shunting part of the blood of the heart-lung-kidney preparation through the head and neck of a dog, the pituitary body of which has previously been removed, is unaccompanied by that inhibition of urine flow and percentual rise in chloride which characterise the operation when the pituitary body is intact.

C.—HISTOLOGICAL EXAMINATION.

The results of histological examination of the perfused kidneys and of the pituitary region in the experiments already recorded may be briefly mentioned.

The kidneys were weighed, examined macroscopically, and two representative pieces immediately placed into weak formalin. Sections were cut and stained with hæmatoxylin and eosin. The main purpose of the histological examination was to show if there were any sign of chronic nephritis present. Molitor and Pick (31) have demonstrated the impotence of pituitrin to inhibit water diuresis in dogs after acute cantharidin poisoning. Starling and Verney (*loc. cit.*) reported one case in which pituitrin had very little, if any, effect on the percentage whilst definitely lowering the absolute amount of chloride eliminated in the urine secreted by the isolated kidney, and in this case the kidney was found to be markedly fibrosed. No signs of inflammation were observed, with the following exceptions:—In the kidney of the experiment described in Protocol 1 there were a few hæmorrhagic spots in the boundary zone which showed microscopically as areas crowded with red and white blood cells. The cortex of the kidney appeared normal. A similar picture was present in the kidney of the experiment summarised in Table IV. In that of the experiment illustrated in fig. 8 there

were present a few small subcapsular wedge-shaped fibrotic areas, the remainder of the kidney, however, presenting a normal histological appearance.

In those experiments in which the pituitary body was removed, the removal was confirmed by taking serial sections of the parts removed and of the subdural contents of the sella turcica, and sections at intervals of 200 μ of the optopeduncular region of the base of the brain. Traces of pars tuberalis tissue were invariably present adhering to the floor of the third ventricle. In the experiment shown in fig. 8 the pituitary region of the mid-brain was also cut serially. No pituitary tissue was left in the sella, and on the floor of the third ventricle were seen the remains of the epithelial portion of the hypophysis, into which there had occurred extravasations of blood. Some of this blood had passed into the infundibulum and was lying free in this region. There was no evidence of injury to the base of the brain in the region around the attachment of the pituitary. A similar histological picture was seen in the section of the pituitary region in the experiment shown in Table IV. A small epithelial fragment was found lying in a small blood clot in the sella turcica. Fragments of epithelial tissue were also seen adhering to the infundibulum: into this a few small hæmorrhages had taken place.

D.—DISCUSSION.

There seems to be only one interpretation to be placed on the experimental results recorded in this paper: pituitrin is contributed by the pituitary body to the blood during its passage through the tissues of the head and neck. Attention may be directed especially to the ingravescent manner in which the reaction of the isolated kidney reveals itself during the period of head perfusion, a phenomenon which is readily interpreted by, and indeed is to be expected from, the increasing number of circuits which the blood makes through the tissues of the head. During this time, then, we must assume that the balance of secretion over destruction is turned in favour of the former. It will have been observed that in those experiments in which the pituitary was removed before switching the head into the circuit there was a small initial inhibition in the rate of urine flow and a small augmentation in the chloride output. This was transitory, however, and the reappearance and augmentation of the polyuria and chloride fall in the time during which the blood was still shunted through the head, showed that even if pituitrin were being continuously secreted during this time by the remaining fragments which inevitably escaped removal, its rate of destruction in the blood and tissues was certainly greater than its rate of formation.

One is not justified, however, in concluding that the pituitary principle

involved in the reaction on the isolated kidney is poured into the blood stream directly. The researches of Thaon (32) showed the absence of a lymphatic network in the pituitary body, and the histological picture gave the impression of the potential passage of any secretory product directly into the capillaries. Later Cooper (33) drew attention to the rich sinusoidal blood supply of the pars distalis of the California ground-squirrel. On the other hand, the high concentration in which active principles are found in the pars posterior and even in the pure hypothalamic tissue (Abel (34)), though by no means proving the activity of these parts in their formation or even in their physiological transport, points to the possibility of their passage through the infundibulum into the third ventricle, as Herring (35) suggests from histological evidence. Dixon (36) states that "samples of normal cerebro-spinal fluid of the dog show every known chemical and physiological action of pituitrin. . . . It contracts the uterus and blood vessels, raises the blood pressure, increases the urinary flow, and under certain conditions causes a secretion of milk." This author showed that the oxytocic principle appeared in the cerebro-spinal fluid of dogs and cats immediately after pituitrin was injected intravenously. The effect could not be obtained after the pituitary body had been destroyed by the cautery. It was not proven, however, that the amount of oxytocic principle present in the cerebro-spinal fluid following intravenous injection of pituitrin was larger than the amount injected. Further, the increase in the oxytocic action of the cerebro-spinal fluid following intravenous injections of ovarian extract was not shown to be dependent on the integrity of the pituitary body. Cushing and Goetsch (37) agree that there is present in the cerebro-spinal fluid a substance which gives the pressor reaction of pituitary extract. Its potency as regards the remaining known physiological reactions of pituitrin appears not to have been investigated. The fact that free exposure of the pituitary body in the experiments recorded in Series III of this paper had apparently no effect on the reaction of the head on the isolated kidney, gives no information as to whether the passage of pituitrin from the pituitary body to the blood is direct or indirect.

In Cow's experiments (38), cats' kidneys were perfused *in situ* with Ringer, and an increased rate of flow from the ureter observed when the organ was switched to a perfusion fluid consisting of a Ringer extract of the pituitary body or a Ringer duodenal extract which had already been perfused through the general circulation. His experiments were concerned with the secretion of a diuretic principle by the pituitary body, and were both in their nature and in the results obtained fundamentally different from those described in this paper. Here we are concerned with the secretion by the pituitary body of an anti-

diuretic and chloride-raising principle, and this secretion we believe to have been experimentally demonstrated.

It must be left to future experiments to decide which of the parts of the pituitary body is or are essentially concerned with its formation. Similarly, it is impossible from these experiments to form any accurate quantitative idea of the amount of pituitrin picked up by the blood. It may be permissible, however, to hazard the impression that has been gained from a comparative mental picture of the effects on the isolated kidney of the passage of the blood through the intact head on the one hand, and that of the actual addition of pituitrin to a similar volume of circulating blood on the other, that the degree of magnitude of the amount contributed by the pituitary to the blood is of the order of 0.01 c.c. of the commercial 17 per cent. extract per 15 to 30 minutes.

It is of interest now that the regulating function of pituitrin on salt and water excretion has been demonstrated to recall some experiments of Eckhard (39) published in 1903. This experimenter stated that piqure of the floor of the fourth ventricle in rabbits leads to non-saccharine polyuria even one to fourteen days after bilateral splanchnotomy, reaching its maximum rate of flow in the first hour and falling from the second hour on. Unfortunately, the chloride content of the urine was not considered. It may well be that the pituitary was involved in this phenomenon, especially since a similar effect followed bilateral ligation of the carotids. The results, however, were interpreted as being caused by stimulation of the kidney "secretion regulating centre" in the medulla by the cerebral anæmia resulting from the diminution in blood supply to the brain.

Whether the secretory phenomenon described in this paper is to be categorised within the province of the first or second group of internal secretory organs towards which attention was directed at the beginning cannot yet be decided. It may be that the abnormal conditions under which secretion was observed in these experiments were just such as to produce the stress necessary for secretory activity to be manifested. On the other hand, it may be—and this we are inclined to believe to be the case judging from the acute effects of removal of the pituitary body from the whole animal—that the secretion of the anti-diuretic and chloride-raising principle is a continuous process, and that the reactions of the kidney are dependent for their physiological conduct on the continuous presence of this principle in the blood stream.

E.—SUMMARY AND CONCLUSION.

1. The isolated kidney of the dog has been used as a means whereby the blood supplying it may be tested as to its content in pituitrin-like substance.
2. When the head and neck of a dog are switched into perfusion-parallel with the isolated kidney (*see* p. 490), the blood picks up during its passage

through the head and neck a substance or substances which inhibit the polyuria of the isolated kidney, augment the urinary chloride output percentually or sometimes absolutely, and diminish the renal blood flow.

3. This result is still obtained after previous exposure of the pituitary body.

4. No such phenomenon occurs as the result of perfusing the pelvis and lower limbs of a dog in parallel with the kidney.

5. Previous removal of the pituitary body abolishes the reaction summarised in 2 above.

An antidiurectic, chloride-augmenting, and vaso-constrictor principle or principles are contributed by the pituitary body to blood during its passage through the head of a dog.

The expenses of this research were defrayed in part out of a grant from the Government Grants Committee of the Royal Society.

F.—PROTOCOLS.

1.

Dogs I and II. Morphia. C. and E. Bled.

Dog III, 11 kilos. C. and E. Chloralose. Prepared for head perfusion. Defibrinated.

Dog IV, 16.5 kilos. Morphia. C. and E. Chloralose. Left kidney prepared.

Dog V, 15 kilos. Morphia. C. and E. Chloralose. Heart-lung. Heart-lung-kidney completed. About 2,500 c.c. blood circulating. Add 2 gms. urea.

Time.	T °C. Kidney.	B.P. Mm. Hg. Kidney.	Blood flow c.c./min. Kidney.	Urine. c.c./15 min.	Urine. NaCl. Mgms. Per cent.
4.36—4.41	37.0	119	272	15.0	160
4.41—4.48	37.0	120	—	15.0	150
4.48—4.57	36.8	120	—	14.0	100
4.58—5.07	36.7	120	272	10.3	160
5.07—5.15	37.1	119	272	7.4	140
5.15—5.25	37.0	121	272	5.1	165

← Switch to head

Temperature and pressure of blood supply to head = 36° C. and 90 mm. Hg. Blood flow through head = 190 c.c./min. Kidney = 71 gms., œdematous, with a few hæmorrhagic spots in boundary zone.

2.

Dogs I and II. Morphia. C. and E. Bled.

Dog III. Morphia. C. and E. Chloralose. Left carotid exposed. Natural respiration Aorta and inferior vena cava prepared for perfusion. Defibrinated.

Dog IV. Morphia. C. and E. Chloralose. Left kidney prepared.

Dog V. Morphia. C. and E. Chloralose. Heart-lung.

Heart-lung-kidney completed. About 2,000 c.c. blood circulating. Add 3 gms. urea.

Time.	T °C. Kidney.	B.P. mm./Hg. Kidney.	Blood flow c.c./min. Kidney.	Hb. Per cent.	Urine c.c./15 min.	Urine NaCl Mgms. Per cent.
3.52—3.59	36.6	127	200	—	7.0	130
3.59—4.06	36.8	127	—	—	7.5	70
4.06—4.11	37.0	127	—	100	8.4	70
4.11—4.22	37.5	129	—	—	8.4	70
4.22—4.30	38.0	131	230	105	7.9	70 ← Switch to legs.
4.30—4.38	38.0	130	—	—	6.8	70
4.38—4.45	38.0	130	230	106	6.7	70

Perfusion pressure to, and blood flow through legs = 100 mm. Hg and 72 c.c./min. respectively, 425 c.c. washed through before blood diverted to venous reservoir. Mercury valve in left carotid adjusted to 95 mm. Hg. Blood gradually disappeared into dog III; at end of experiment about 600 c.c. only circulating.

Kidney = 62 gms. Urines, all clear, no Hb.

Hb. in blood of dog III = 116 per cent.

3.

Dogs I and II. Morphia. C. and E. Bled.

Dog III, 14 kilos. Morphia. C. and E. Chloralose. Prepared as in Protocol 2, with additional exposure of sciatic nerves.

Dog IV, 16 kilos. Morphia. C. and E. Chloralose. Left kidney prepared.

Dog V, 7 kilos. Morphia. C. and E. Chloralose. Heart-lung. Heart-lung-kidney completed. About 2,500 c.c. blood circulating. 5 gms. urea added.

Time.	T °C.	B.P. mm Hg. Kidney.	Blood flow c.c./min. Kidney.	Hb. Per cent.	Urine. c.c./15 min.	Urine NaCl Mgm. Per cent.
4.55—5.21	37.5	127	190	—	1.3	110
5.21—5.31	37.5	128	—	—	4.7	75
5.31—5.41	37.5	125	180	100	5.7	80
5.41—5.53	37.5	126	—	—	7.8	110
5.53—6.03	37.5	130	180	100	7.8	110 ← Switch to legs.
6.03—6.13	37.5	128	190	—	6.8	110

Legs denervated just before switching them into the circuit. 550 c.c. washed through before blood diverted to venous reservoir. Mercury valve in left carotid adjusted to 95 mm. Hg. Kidney = 69 gms. Hb, per cent. in blood of dog III at 5.50 = 119 per cent. Blood flow through, and perfusion pressure to, legs = 250 c.c./min. and 100 mm. Hg respectively.

4.

Dog I. C. and E. Bled.

Dog II, 9 kilos. C. and E. Chloralose. Tracheal cannula. Pituitary exposed. Artificial respiration. Chest opened and vessels prepared for head perfusion. B.P. from left femoral artery.

Dog III, 13 kilos. Morphia. C. and E. Chloralose. Heart-lung. Right kidney of dog II excised and perfused by heart-lung. Urine started to flow immediately. 3 gms. urea added.

Time.	T °C. Kidney.	B.P. mm. Hg. Kidney.	Blood flow c.c./min. Kidney.	Hb. Per cent.	Urine. c.c./15 min.	Urine. NaCl Mgm. Per cent.
3.10—3.24	36.5	180	—	—	4.5	460
3.24—3.33	36.2	177	155	100	8.3	250
3.33—3.39	37.0	177	190	—	7.5	270
3.39—3.48	37.0	176	180	—	9.6	250
3.48—4.04	37.0	171	170	—	11.0	230
4.04—4.10	36.0	176	160	106	13.8	240 ← Switch to head.
4.10—4.16	37.0	181	160	—	15.6	360
4.16—4.27	37.4	177	170	115	6.0	440
4.27—4.42	—	176	160	117	2.9	500

Blood flow through head = 215 c.c./min. Perfusion pressure to head = 140 mm. Hg.

5.

Dog I. C. and E. Bled.

Dog II, 10.7 kilos. C. and E. Chloralose. Prepared as in Protocol 4.

Dog III. C. and E. Chloralose. Bled 300 c.c., 350 c.c. normal saline i.v. Left kidney prepared.

Dog IV. Heart-lung.

Heart-lung-kidney prepared. Urine started to flow immediately. 3 gms. urea added.

Time.	T °C. Kidney.	B.P. mm. Hg. Kidney.	Blood flow. c.c./min. Kidney.	Urine. c.c./15 min.	Urine. NaCl Mgms. Per cent.
3.58—4.08	34.0	174	—	8.4	750
4.08—4.15	34.0	179	—	12.6	570
4.15—4.22	35.0	176	—	12.2	480
4.22—4.30	36.0	174	245	12.6	340
4.30—4.39	36.5	172	260	11.4	330
4.39—5.00	—	164	—	8.9	250
5.00—5.13	36.0	158	175	3.6	480 ← Switch to head.
5.13—5.34	37.0	155	180	2.4	530

Temperature of blood supply to head = 37° C.

6.

Dogs I and II. Bled under C. and E.

Dog III, 12.5 kilos. C. and E. Chloralose 1.4 gr. i.v. *Pituitary removed.* Chest opened and vessels prepared for head perfusion. Defibrinated.

Dog IV, 11 kilos. Morphia. C. and E. Chloralose. Left kidney prepared.

Dog V, 12.5 kilos. Morphia. C. and E. Chloralose. Heart-lung prepared.

Heart-lung-kidney completed. 4 gms. urea added.

Time.	T °C. Kidney.	B.P. Mm. Hg.	B.F. c.c./min.	Urine. c.c./15 Min.	Urine. NaCl Per cent.
6.54—7.00	36.0	148	—	4.7	170
7.00—7.10	36.0	134	—	4.5	100
7.10—7.22	36.0	132	125	6.7	80
7.22—7.28	36.0	128	143	6.5	70 ← A.
7.28—7.35	36.0	128	—	5.8	70
7.35—7.43	36.0	132	134	5.3	60

At A head, neck and right fore-limb of dog III switched into perfusion-parallel with the kidney.

Urines clear: no Hb. Kidney = 36 gms Blood flow through head = 215 c.c./min.

Head blood pressure = 100 mm. Hg.

REFERENCES.

- (1) Claude Bernard, 'Nouvelle Function du Foie,' J. B. Ballière, Paris (1853).
- (2) Anrep and Daly, 'Roy. Soc. Proc.,' B, vol. 97, p. 450 (1925).
- (3) Murray, 'Brit. Med. Journ.,' vol. 2, p. 796 (1891).
- (4) Banting and Best, Communication to the Academy of Medicine (Toronto), February 7, 1922. Cited from Macleod, 'Physiol. Reviews,' vol. 4, p. 21 (1924).
- (5) Staderini, 'Arch. di Fisiol.,' vol. 8, p. 128 (1910).
- (6) Tilney, 'Internat. Monatschr. f. Anat. u. Phys.,' vol. 30, p. 258 (1913).
- (7) Vassale and Sacchi, 'Riv. speriment di Freniatria,' vol. 18, p. 525 (1892).
- (8) Pironne, 'Riforma Medica,' vol. 19, pp. 197 and 205 (1903).
- (9) Gemelli, 'Arch. Ital. de Biol.,' vol. 50, p. 157 (1908).
- (10) Paulesco, 'Journ. de Phys. et de Path. général,' vol. 9, p. 441 (1907).
- (11) Crowe, Cushing and Homans, 'Johns Hopkins Hosp. Bull.,' vol. 21, p. 127 (1910).
- (12) Dean Lewis and Matthews, 'Trans. Chicago Path. Soc.,' vol. 9, p. 16 (1913).
- (13) Frank, 'Berlin Klin. Wochensch.,' vol. 49, p. 393 (1912).
- (14) Von den Velden, *Ibid.*, vol. 50, p. 2083 (1913).
- (15) Camus et Roussy, 'Compt. Rend. d. l. Soc. d. Biol.,' vol. 75, pp. 483 and 628 (1913).
- (16) Houssay, 'Endocrinology,' vol. 2, p. 94 (1918).
- (17) Leschke, 'Zeitschr. f. Klin. Med.,' vol. 87, p. 201 (1919).
- (18) Bailey and Bremer, 'Arch. of Int. Med.,' vol. 28, p. 773 (1921).
- (19) Larson, Weir and Rowntree, 'Journ. Pharm. and Exper. Therap.,' vol. 17, p. 333 (1921).
- (20) Maranon, 'Endocrinology,' vol. 5, p. 159 (1921).

- (21) Blumgart, 'Arch. of Int. Med.,' vol. 29, p. 508 (1922).
 - (22) Camus and Roussy, 'Compt. Rend. d. l. Soc. d. Biol.,' vol. 83, p. 1578 (1902).
 - (23) Camus and Roussy, 'Journ. de Phys. et de Path. général.,' vol. 20, pp. 509 and 535 (1922).
 - (24) Bailey, 'Ergeb. der Phys.,' vol. 20, p. 162 (1922).
 - (25) Starling and Verney, 'Roy. Soc. Proc.,' B, vol. 97, p. 321 (1925).
 - (26) Starling, 'Journ. Phys.,' vol. 44, p. 206 (1912).
 - (27) Millard Smith, 'Journ. Biol. Chem.,' vol. 45, p. 437 (1921).
 - (28) Anrep and Starling, 'Roy. Soc. Proc.,' B, vol. 97, p. 463 (1925).
 - (29) Reford and Cushing, 'Johns Hopkins Hosp. Bull.,' vol. 20, p. 105 (1909).
 - (30) Dandy and Goetsch, 'Amer. Journ. Anat.,' vol. 11, p. 137 (1911).
 - (31) Molitor u. Pick, 'Arch. f. exp. Path. u. Pharm.,' vol. 101, p. 169 (1924).
 - (32) Thaon, 'Compt. Rend. d. l. Soc. d. Biol.,' vol. 62, p. 714 (1907).
 - (33) Cooper, 'Amer. Journ. Anat.,' vol. 26, p. 185 (1919).
 - (34) Abel, 'Johns Hopkins Hosp. Bull.,' vol. 35, p. 305 (1924).
 - (35) Herring, 'Quart. Journ. Exper. Phys.,' vol. 1, p. 121 (1908).
 - (36) Dixon, 'Journ. Phys.,' vol. 57, p. 129 (1923).
 - (37) Cushing and Goetsch, 'Amer. Journ. Phys.,' vol. 27, p. 60 (1910).
 - (38) Cow, 'Journ. Phys.,' vol. 49, p. 441 (1914).
 - (39) Eckhard, 'Zeitschr. f. Biol.,' vol. 44, p. 407 (1903).
-

OBITUARY NOTICES
OF
FELLOWS DECEASED.

CONTENTS.

	PAGE
SIR ARCHIBALD GEIKIE (with portrait)	i
THOMAS GEORGE BONNEY (with portrait)	xvii
SIR WILLIAM MADDOCK BAYLISS (with portrait)	xxvii
ARTHUR DENDY (with portrait)	xxxiii
FRANK EVERS BEDDARD	xxxvi
JAMES SYKES GAMBLE (with portrait)	xxxviii
SIDNEY HARRIS COX MARTIN	xliv
JAMES FAIRLIE GEMMILL	xl v



Archibute

SIR ARCHIBALD GEIKIE—1835-1924.

SIR ARCHIBALD GEIKIE was elected a Fellow of the Society in 1865, at the age of 29 years, and at the time of his death, in 1924, he was the senior Fellow, and hence the "father" of the Society. Throughout this long series of years he was devotedly attached to the Society and most anxious to promote its welfare and further its activities in all possible directions, and the Royal Society is much indebted to him for the services he rendered to it during the periods he acted as one of its officers. He served on the Council first from 1885 to 1887, and was Foreign Secretary from 1889 to 1893. In 1903 he was elected Secretary on the biological side in succession to Sir Michael Foster, and had as colleague on the physical side his friend Sir Joseph Larmor, the presidents during his term of office as Secretary being, Sir William Huggins and Lord Rayleigh.

During the later years of the nineteenth century the work of the Society had undergone considerable expansion both on the physical and on the biological side. Thus, the part played by the Society in such undertakings as the International Catalogue of Scientific Literature, the Association of Academies, the National Physical Laboratory, and the investigations into certain tropical diseases, considerably increased not only the range of the activities of the Society, but also added considerably to the work of the Officers, Fellows, and staff. Geikie, throughout his tenure of office, took the greatest interest in the work of the Society as a whole, and his outlook was always a wide one, although as Secretary his activities were mainly concentrated on the biological side. One of the most characteristic features of his work was the interest he took in all biological questions, botanical, zoological, and physiological. He never confined his interest to the more special branches of knowledge that he had made his own.

During Sir Michael Foster's tenure of the Secretaryship, and whilst Mr. Joseph Chamberlain was Secretary of State for the Colonies, arrangements had been made, in consultation with the Colonial Office, for the Royal Society to undertake the supervision and control of a series of inquiries into the causation and nature of important maladies affecting the welfare of our tropical Dominions and dependencies, *e.g.*, malaria, Malta fever, sleeping sickness. Valuable results have been obtained by the successive commissions sent out under the auspices of the Tropical Diseases Committee of the Royal Society. Geikie took the greatest possible interest in this work, not only from the actual scientific interest of the questions involved and the results obtained, but also from the point of view, that the Royal Society in super-

vising and controlling such work for the assistance of the Government, was doing work for which it was exceptionally fitted, and fulfilling a most important function in promoting the welfare of the Empire.

Like Foster, Geikie held the view that all such work undertaken for the Government should be rigorously confined, as it was, to the investigation of definite scientific problems, and not extended to either administrative matters, or to supervising methods of medical treatment of given diseases. This work often entailed a considerable burden on the Officers and Fellows of the Society, and also some increase in the actual expenditure of the Society; but Geikie felt that the Royal Society, as the foremost scientific society, should be willing to undertake such work, and that it was specially fitted to carry it out to a successful conclusion. On relinquishing the secretaryship to become the President of the Society, he maintained the closest possible touch with the Tropical Diseases Committee, and rarely missed taking the chair at its meetings. Certainly the Fellows of the Society interested in physiological and pathological questions are indebted to him for the wide interest he felt in, and the sympathy he extended to, their investigations and work.

In his anxiety that the Society should continue to hold its place as the foremost Scientific Society, he was always wishful that it should contain within its ranks men distinguished in all branches of natural knowledge, and more especially that it should include amongst its fellows, those who had gained distinction from their researches, although not following a scientific career as a profession. The British Empire has long been noted for the number of men who have pursued scientific studies and gained distinction by the results achieved, notwithstanding the fact that such studies have either formed their relaxation, or else have been pursued solely out of the interest evoked by them. Such men have often been miscalled amateurs, and the Royal Society has always contained such men amongst its fellows. Geikie was greatly impressed with the necessity of maintaining this custom, as he was convinced that it gave the Society a breadth of view that otherwise it might not have. He even went further, and often regretted that the old custom of including amongst the Fellows men who had obtained distinction in walks of life other than those actually included under the term natural knowledge, was not more frequently followed. He felt strongly that it was only by these means that the Society would maintain the status, it was rightfully entitled to, as representative of learning. In this connection it may be recalled that it was during his presidency that arrangements were made whereby the recently founded British Academy was enabled to use the apartments of the Society as a meeting place.

Geikie was a very efficient officer of the Society, spending much time in the offices of the Society and making himself familiar with all the details of the work that falls to the lot of the officers. He was a pleasant colleague to work with, and had usually clearly cut and defined views on the various questions

that came up for decision by the Officers and Council, and thus the business of the Society was conducted with dispatch under his auspices.

The activities of the Society have for long been somewhat hampered by the inadequacy of the funds at its disposal, and Geikie, like other Presidents of the Society, often alluded in his addresses and public utterances to the necessity of more liberal endowments if the Society was to fulfil its functions in the advancement of natural knowledge. It was a source of gratification to him in his last years that several munificent benefactions were received by the Society, thereby enabling it to endow Research Professorships in several branches of Science.

Geikie not only made himself familiar with other branches of Science outside the range of his own speciality, but, in addition, he had a well-developed literary and artistic side. His literary ability is well seen in his numerous writings dealing with a considerable variety of subjects, some biographical, several relating to his beloved Scotland, *e.g.*, *Scottish Scenery* and *Scottish Reminiscences*, and several classical, more especially relating to Roman family life, Horace's villa, etc. He was at one time fond of sketching, especially amidst the Western Isles, and the present writer well remembers the graphic manner in which he described these scenes, and how he had wished to follow the career of an artist to place them on canvas. For many years he was in the habit of yachting on the West Coast of Scotland, and was familiar not only with the scenery, but also with the folk-lore and traditions of the islanders. He had a fund of Scottish stories and was an admirable raconteur, telling his stories with a keen relish of their humour. Many of them may be found in his well-known *Scottish Reminiscences*.

The 250th Anniversary of the foundation of the Royal Society was celebrated in 1912 during his tenure of the presidency, and he not only took the greatest interest in the various ceremonies and functions that marked the anniversary, but also played a very active part in the work of the Officers and staff in making all the arrangements necessary for the celebration. Further, the Society is indebted to him for compiling and editing a new and enlarged edition of the *Record of the Royal Society*, and it was at his instigation that a facsimile of the ancient Charter Book was prepared. Geikie took a very special interest in all that concerned the life of the Royal Society in the past, and one of the best illustrations of this and of his literary activities is to be found in the history of the Royal Society Club that he published in 1917, after he had relinquished the presidency of the Society, under the title of the *Annals of the Royal Society Club*. This is a very complete and accurate history of the doings of the club from its foundation in 1743 up to the year 1902, and contains much information of the manners and customs of the times and many anecdotes illustrating the characteristics of some of the more prominent of the early Fellows of the Society. Geikie was a member of the club from 1890 to his death, and was a very constant attendant and a great believer in the humanising influence of

social meetings, especially those of a dining club with such ancient traditions as those of the Royal Society Club.

Geikie received the honour of Knighthood in 1891 and that of K.C.B. in 1907, and in 1913 the Order of Merit was conferred on him and he also became Officier de la Légion d'Honneur.

Geikie married on August 10th, 1871, Alice Gabrielle Anne Marie Pignatel, who died on January 21st, 1916. His family consisted of one son, Roderick, who died on December 6th, 1910, and three daughters; one of these died on February 23rd, 1915. Sir Archibald Geikie died on November 10th, 1924.

J. R. B.

The scientific career of Sir Archibald Geikie was one of great distinction. Joining the Geological Survey in 1855, he became Director-General of the Geological Survey of the United Kingdom in 1882; President of the Geological Society, London, 1890-92; and was selected by the Council of that Society for a second term of office, to preside at the Centenary Celebrations in 1907. Honours were showered upon him by scientific societies at home and abroad and by Universities; these were crowned by the award of the Order of Merit in 1914.

Born in Edinburgh in 1835, and educated at the High School and University, he derived inspiration from the classic surroundings—the scene of the fierce controversy between the Huttonians and Wernerians at the beginning of last century. His finding of fossils in the Burdiehouse limestone in his boyhood marked the first stage in his field experience. He roamed over Arthur's Seat and the Pentland Hills with Charles Maclaren's volume on 'The Geology of Fife and the Lothians' as his guide. Not content with excursions in the neighbourhood of Edinburgh, he went to Arran with Ramsay's account of the geology of that island in his hand to examine the metamorphic rocks and palæozoic strata pierced by the granite masses in the north of the island. This expedition was followed, in his eighteenth year, by a visit to Skye, where he began the mapping of the Lias, in the district of Strath, after a careful study of Macculloch's volume on 'The Geology of the Western Islands.'

All these excursions sprang from his own initiative. They showed that he was clearly destined for a geological career, if such could be obtained. Two articles which he wrote on his work in Arran secured an introduction to Hugh Miller, then living in Edinburgh, which led to a close friendship that lasted till the tragic end of Hugh Miller's career. The young geologist paid frequent visits to that home with his specimens, his maps, and notes, receiving in return great encouragement to continue the work. Soon after this introduction, Hugh Miller was asked by Sir Roderick Murchison, who had been appointed Director-General of the Geological Survey, to recommend a young geologist

to carry on the mapping in East Lothian, begun by Professor Ramsay in 1854. Archibald Geikie was recommended and he joined the staff in his twentieth year.

He made the acquaintance of other scientific men in Edinburgh at that time, who were impressed with his enthusiasm for geological research. Dr. George Wilson, Lecturer on Chemistry in the extramural school and Director of the Science and Art Museum, in whose laboratory he did chemical analyses, followed his early progress with great interest. Robert Chambers, Professor Fleming, one of the able naturalists of that period, and Professor James David Forbes, whose work among the glaciers of the Alps and Norway is familiar to geologists, befriended him. Forbes, who was Secretary of the Royal Society of Edinburgh for twenty years, appealed to him to contribute papers to the Society, for he regretted the decline of geological research in Scotland since the days of Hutton, Hall and Playfair. Archibald Geikie responded cordially to this appeal in later years by communicating his best papers to this Society.

From the point of view of worldly advancement, Murchison was his greatest benefactor. In 1860, after a few years' experience of field work in the lowlands, the young geologist was requested by Murchison to accompany him in the North-west and Central Highlands to determine the order of succession of the rocks in those regions. From these traverses arose an intimate friendship, which lasted till the close of Murchison's life in 1871. Through Murchison's influence he was appointed first Director of the Geological Survey in Scotland in 1867. When Murchison offered to share the endowment of the new Chair of Geology in Edinburgh University, he stipulated with the Government that Archibald Geikie should be the first Professor.

The areas fixed by Professor Ramsay, then Director of the Geological Survey of Great Britain, to be mapped by Archibald Geikie in his early official career, gave him great gratification. His colleague was H. H. Howell, a coal-field expert, and after completing the survey of the low ground in the county of Haddington, they moved westward to Midlothian. To Geikie was assigned the area west of the coalfield, ranging from Arthur's Seat and the Pentland Hills to the Bathgate Hills, and northwards into Fife. His experience in those areas determined the lines of research in which he did his best work. The evidence bearing on the glaciation of the region, the development of the Old Red Sandstone formation in the Pentland Hills, and the occurrence of volcanic rocks on different geological horizons, suggested fields of investigation that might bear rich fruit.

In his early survey days, in common with the prevalent opinion of that time, he attributed the polishing and grooving of the rocks to the action of currents and icebergs, but under the influence of Professor Ramsay, who had already described the vanished glaciers of North Wales, he adopted the view, propounded by Agassiz during his visit to Scotland in 1840, that these phenomena were due to the action of land ice. In 1862 he contributed to the Glasgow Geological Society a valuable paper on 'The Glacial Drift of Scotland' (published in

1863), which helped to place the investigation of these deposits on right lines. He showed how the rock striations radiated from the main mountain ranges ; he described the local character of the boulder-clay, its relation to the underlying rocks, the direction of transport of the materials, its striated pavements, the intercalated deposits of sand and gravel—observations which have stood the test of time. From these phenomena he inferred that during the earlier part of the glacial period Scotland was covered with an ice sheet after the manner of Greenland, that the boulder-clay was a product of this ice-sheet, and that the stratified beds in the Till, marked periods of lessened severity of climate when the ice retired for some distance. He described the inland stratified drift and the fossiliferous marine shelly clays of the Clyde basin, so admirably worked out by Smith of Jordanhill. He suggested that the moraines in the upland valleys indicated the gradual shrinkage of the ice-sheet into local glaciers, and the final disappearance of the ice.

Availing himself of the researches of Smith of Jordanhill, Edward Forbes, T. F. Jamieson and others, he appended to this paper a list of organic remains from the glacial deposits of Scotland.

This paper proved of great service to British geologists at that time, as it gave an outline of the conditions which probably prevailed during the glacial period in this country, and suggested the lines along which investigation should proceed. It held the field till the publication in 1874 of the well-known volume, 'The Great Ice Age,' by his younger brother, Professor James Geikie.

As the Scottish survey work advanced, it fell to the lot of Archibald Geikie to map in detail large areas of the Old Red Sandstone in Midlothian, Lanarkshire, Ayrshire, and in the counties of Fife, Perth and Kinross. He also examined the districts surveyed by other members of the staff south of the Grampians. Throughout this region it had been proved that this formation consisted of two divisions, an upper and lower, separated by a marked unconformability, and characterised by different fish-faunas.

North of the Grampians, however, Murchison adopted a triple classification by introducing a middle division, composed of the Caithness flagstones. From their lithological characters and fossil contents, he maintained that this flagstone series belonged to a different division of the formation. He argued that the fish-fauna found in this series differed in important points from that occurring in the flagstones, sandstones and shales of Lower Old Red Sandstone age in Forfarshire. It was therefore of younger date. He also suggested that the Caithness flagstones might have been laid down during the long interval separating the lower and upper divisions south of the Grampians.

Hugh Miller and Dr. Malcolmson had proved the occurrence of the upper division with its characteristic fish-remains in the basin of the Moray Firth, and they further showed that the ichthyolites found in the lower beds in that region resembled those met with in the flagstones of Caithness and Orkney.

Such was the position of enquiry when Geikie began a series of traverses

in the basin of the Moray Firth, Caithness, Orkney and the Shetland Isles. In 1878 he communicated to the Royal Society of Edinburgh an elaborate paper on the 'Old Red Sandstone of Western Europe,' which embodied the results of these traverses. It was intended to be the first of a series, descriptive of the other areas occupied by this formation in the British Isles, but his removal to London in 1882 prevented him from carrying out this intention. The other areas were dealt with at a later date in his treatise on the 'Ancient Volcanoes of Great Britain,' with special reference to the records of volcanic action associated with them.

One of the special features of this communication is the vivid description of the geographical changes in Western Europe that followed the marine conditions of Silurian time. Adopting the theory of the lacustrine origin of the Old Red Sandstone suggested by Fleming and Godwin Austin, and supported by Rupert Jones on palæontological evidence, and by Professor Ramsay on lithological grounds, he held that in North-West Europe the Silurian sea gave place to continental conditions with large inland lakes. He defined the areas of the various basins of deposit of the Old Red Sandstone in the British Isles, and gave them the following names:—(1) Lake Orcadie, including the extensive region north of the Grampian range and stretching north to the Shetland Isles; (2) Lake Caledonia or the Mid-Scottish Basin between the Grampian Highlands and the Southern Uplands; (3) the Lake of Lorne extending from the south-east of Mull to Loch Awe; (4) Lake Cheviot, including a part of the south-east of Scotland and north of England; (5) the Welsh Lake, bounded on the north by the Cambrian and Silurian high grounds. its southern and eastern extension being obscured by later formations.

The deposits in Lake Orcadie were grouped by him in two divisions; a lower, comprising the Caithness flagstone series, and an upper, composed of false-bedded sandstones and marls with its characteristic fish-fauna, resting uncomformably on the lower as in the midland valley of Scotland. He pointed out that Murchison's lower division in Caithness consisted merely of the thick accumulation of sandstones, breccias and conglomerates that underlie the Caithness flagstone series.

The distinctive feature of the paper was his correlation of the Caithness flagstone series (Murchison's middle division) with the true Lower Old Red Sandstone south of the Grampians. He admitted the marked differences in the lithological characters and fossil contents of the deposits in the two areas, but he contended that the palæontological distinctions are probably not greater "than the contrast between the ichthyic faunas of adjacent but disconnected water basins at the present time."

Much new information bearing on the distribution and field relations of these deposits was embodied in this communication. The main features in the geological structure of Caithness were indicated; the great overlap at Reay in the north of that county, where the higher members of the flagstones

rest unconformably on the crystalline schists, was clearly established; his suggestion that the fish-bearing bands and associated strata in the Moray Firth basin are the equivalents of part of the higher portion of the Caithness flagstone series has been confirmed by the later detailed mapping of the Geological Survey. He also recorded for the first time the unconformability between the Upper Old Red Sandstone and the Caithness flagstones visible on the west coast of the island of Hoy, Orkney.

In accordance with his classification the Caithness flagstone series and associated strata were grouped with the lower division of the system in the official publications of the Geological Survey. But after his retirement from the Survey, the palæobotanical evidence threw new light upon the problem. The researches of Dr. Kidston and Mr. P. Macnair showed that the assemblage of plants found in the Caithness flagstones differed from those met with in the Lower Old Red Sandstone south of the Grampian Chain, and ought to be referred to a middle division. The triple classification based upon the plants harmonised with that advanced by Dr. Traquair, based upon the fishes. The value of this evidence was appreciated by the Geological Survey in 1902, and the three-fold grouping of the system has been adopted in the official publications since that date.

Turning now to volcanic geology we enter a sphere of research in which Archibald Geikie laboured with great success. This branch of geological enquiry roused his enthusiasm, and led him to study volcanic phenomena in the British Isles and other lands. As his knowledge of volcanic activity in different geological periods increased, he showed great aptitude in modifying his opinions in accordance with fresh evidence.

In mapping the areas assigned to him in Mid-Lothian, West Lothian and Fife in his early official life, he frankly acknowledged his obligations to the clear descriptions of Charles Maclaren, whose work was far in advance of its time. These descriptions proved the occurrence of volcanic action in Old Red Sandstone and Calciferous Sandstone time. His own field work in the Bathgate Hills showed that volcanoes were active at intervals during the Carboniferous Limestone period on higher horizons than those of the Arthur's Seat volcano. He recorded the prevalence of later intrusive dykes traversing the Carboniferous strata. From his own researches and the work of other investigators, he prepared a paper on the "Chronology of the Trap Rocks of Scotland," published in the 'Transactions of the Royal Society of Edinburgh' in 1861. In the map illustrating this communication the Trap Rocks are referred to the Old Red Sandstone, Carboniferous, Oolitic and Tertiary periods. In accordance with the classification of Edward Forbes, the extensive basaltic plateaux of the West Highlands were assigned to the Jurassic period, except the lavas at Ardtun, in Mull, which are associated with the well-known leaf beds described by the Duke of Argyll. This grouping was corrected in an important paper that appeared in the 'Proceedings of the Royal Society of

Edinburgh' in 1867, in which he suggested that the basaltic plateaux, extending from the North of Ireland, along the West Coast of Scotland to the Faroe Islands and Iceland, were all erupted probably during the Tertiary period. Even at that date he emphasised the importance of the system of intrusive dykes, which, in his opinion, was possibly the most striking manifestation of Tertiary volcanic activity.

While mapping the volcanic area in Fife between Burntisland and the Saline Hills, he visited the Auvergne in Central France, with its extinct but recent volcanoes, to increase his knowledge of volcanic phenomena. In 1868 he visited the volcanic district of the Eifel in Germany, and in 1870, at the request of Poulett-Scrope—author of the well-known volume on 'Geology and Extinct Volcanoes of Central France'—he undertook an examination of the volcanic districts of Southern Italy and the Lipari Islands. He was impressed by the contrast between the comparatively low craters of the Phlegrean Fields, where the eruptive materials consist mainly of tuffs with few lava flows, and the great crater wall of Monte Somma, whose lavas are piled on each other to a great height and pierced by innumerable vertical dykes filling fissures made at successive eruptions. An attack of malarial fever prevented him from carrying out his intention of examining the Lipari Islands.

Another stage in his pursuit of the study of volcanic phenomena in Scotland is marked by an important paper contributed to the Royal Society of Edinburgh in 1879, on the "Carboniferous Volcanic Rocks of the Firth of Forth Basin—their Structure in the Field and under the Microscope."

As his field work proceeded in the Lothians and Fife, he felt the necessity of applying the microscope to the study of the igneous rocks in order to gain definite knowledge regarding their internal composition and structure. He realised that much new light might be thrown on the history of volcanic action in this region by this field of enquiry.

In his historical sketch, he notes with pleasure that the igneous rocks of the Edinburgh district furnished Hutton with the evidence whereby he established the igneous origin of "whinstone" (basalt) and led to the famous experiments of Hall which laid the foundations of experimental geology.

The volcanic masses were grouped by him in four sub-divisions: (1) Necks or Vents; (2) Intrusive Sheets and Dykes; (3) Contemporaneous Lavas; (4) Tuffs. A notable feature of this communication was the prominence given to the numerous necks or vents occurring in the Lothians, Fife and Stirlingshire, from which proceeded showers of ashes and sheets of lava. Most of them were regarded as belonging to different stages of Lower Carboniferous time, while others were supposed to be later than the folding and faulting of the Carboniferous sediments, and were referred to the Permian period. The occurrence of these vents and their wide distribution is one of the valuable contributions made by Archibald Geikie to Scottish volcanic geology.

In the petrographical part of this paper he acknowledges Allport's researches on the Carboniferous dolerites round Edinburgh and gives an outline of his own investigations. Subsequent petrographical work has shown that he did valuable pioneer work in this branch of inquiry, for he was the first to describe many important features of the microscopic characters of these rocks.

Archibald Geikie's discovery of proofs of volcanic action during Permian time in Scotland is of special interest. While mapping the Mauchline district in Ayrshire he recorded a series of contemporaneous lavas and tuffs underlying the Permian sandstones of that region, and forming a ring of higher ground between the Carboniferous and Permian sediments. This discovery was announced in the 'Geological Magazine' in 1866. Similar types of lava, were mapped by him in the Thornhill basin in 1868, where they are also associated with Permian sandstones. He laid special emphasis on the occurrence of volcanic necks in excellent preservation in Ayrshire, which he referred to this period. Some of the smaller ones rise through the ring of Permian lavas, while others occur in the upper division of the Coal Measures. They are filled with agglomerate, pierced in some cases by igneous intrusions.

In describing the volcanic phenomena of Permian age, he suggested that the great series of volcanic vents in East Fife probably belonged to this period. He also suggested that the coarse agglomerates of the Arthur's Seat volcano, and the associated igneous rocks, which, in his opinion, marked a second period of volcanic activity, were probably erupted during Permian time. But the subsequent detailed mapping of the Geological Survey led him to accept Maclaren's later interpretation, confirmed by Professor Judd, that these coarse agglomerates occurred within the vent from which the lavas and tuffs of the hill had been discharged during one period of volcanic action in Lower Carboniferous time.

Another great opportunity of extending his knowledge of volcanic phenomena occurred to Archibald Geikie in 1879. He had arranged to give a course of lectures at the Lowell Institute, Boston, in the autumn of that year, and he resolved to spend the summer in traversing the extensive lava fields, drained by the Snake River, in Idaho, on the Pacific slope of the United States. The vast floods of lava in that region, with no visible cones and craters, were explained by Richthofen as being due to fissure eruptions. Hitherto Geikie had regarded the Scottish volcanic plateaux as having issued from local vents, but this visit widened his conceptions. The great lava plain looked as if it "had been filled with molten rock which had kept its level and wound in and out along the bays and promontories of the mountain slopes as a sheet of water would have done." The Snake River has cut a gorge through this plain, which exposes a succession of sheets of basalt to a depth of several hundred feet. No central cone from which these lavas might have been erupted was visible, only a few cinder cones of secondary origin appeared at wide intervals on the

basaltic plain. The suggestion then occurred to him that the Tertiary volcanic plateaux of Western Europe might have had a similar origin.

Inspired by this conception, he revisited the West Highlands at intervals for several years to continue his investigations among the Tertiary igneous rocks. He was also impelled to do so by the remarkable interpretation of the volcanic history of that region advanced by Prof. Judd. In a paper communicated to the Geological Society on 'The Ancient Volcanoes of the Highlands' in 1874, Prof. Judd described the basal wrecks of five great extinct Tertiary volcanoes (Skye, Mull, Rum, Ardnamurchan and St. Kilda); the one in Mull was estimated by him to have reached a height of 14,500 feet. They indicated three periods of volcanic activity. The first was marked by the extrusion of acid lavas and tuffs connected with plutonic masses of granite, the second by basaltic lavas and tuffs related to deep-seated masses of gabbro, and the third by the discharge of lavas from small sporadic cones after the great central volcanoes had become extinct.

At last, after a quarter of a century of intermittent labour in this subject, Archibald Geikie presented, in 1888, to the Royal Society of Edinburgh his great memoir on 'The History of Volcanic Action during the Tertiary Period in the British Isles.' In the preparation of this monograph he acknowledges the assistance he received from several of his colleagues. A prominent feature of it is the elaborate description of the system of basic dykes, the importance of which he recognised early in his career. His main conclusions may thus be briefly summarised.

Owing to enormous horizontal tension, a series of more or less parallel fissures arose in Tertiary time in a tract of country, including the north of England and Ireland, the southern half and the west coast of Scotland—a total area of about 40,000 square miles. Molten material rose up these fissures, thereby giving rise to the numerous basic dykes which are the distinctive feature of the volcanic region. The basalt plateaux are supposed to be due to streams of lava issuing from these fissures and from vents occurring along these lines of weakness. After these sheets of lava had accumulated to a great thickness, they were injected by laccolitic masses of gabbro, and sills and veins of dolerite. At a later period the gabbros and basalt lavas were alike disrupted and pierced by acid igneous rocks, ranging from granites and granophyres to porphyries and felsites. Crustal movements again ensued whereby another series of fissures was established, now filled with basic dykes that traverse alike the basalt plateaux, the later gabbros, granophyres and granites.

It is interesting to observe that these main conclusions were confirmed by Dr. Harker in the course of his detailed mapping of the central mountain group of Skye for the Geological Survey. But from the recent exhaustive memoir issued by the Geological Survey on the 'Tertiary and Post-Tertiary Geology of Mull, Loch Aline and Oban,' it is evident that the volcanic history of Mull is much more complicated than Sir A. Geikie or Professor Judd imagined. Mull

is regarded as a volcanic centre of extreme complexity which "has repeatedly served as a focus of fissure eruptions ; but it is doubtful whether the lavas still spared by erosion are not, in the main, the products of a central volcano, an idea always linked with the name of Professor Judd."

His last contribution to this branch of geology was his comprehensive treatise on the 'Ancient Volcanoes of Great Britain,' which appeared in 1897. It presented a summary of the knowledge then ascertained regarding these volcanoes, and embodied the results of his own researches and of others who had worked at volcanic problems in the field and in the laboratory. It traversed a wide field of enquiry, for the opening chapters of the treatise are devoted to the discussion of the general principles and methods of investigation of volcanic phenomena, which are followed by detailed descriptions of the proofs of volcanic activity in Britain ranging from pre-Cambrian to Tertiary time.

In the traverses with Murchison in the North-West and Central Highlands in 1860, Archibald Geikie had to deal with problems of fundamental importance connected with the geology of the Highlands. The researches of Macculloch, Murchison and Sedgwick, Hay Cunningham and Hugh Miller, showed that the belt of quartzites and limestones in the West of Sutherland and Ross are succeeded eastwards by metamorphic rocks that stretch across the Great Glen to the eastern border of the Highlands. Accepting Salter's determination of the fossils found in these limestones by Mr. C. W. Peach, Murchison regarded these strata as Silurian (now known to be Cambrian by Mr. A. Macconochie's discovery of the *Olenellus* fauna in the Fucoid beds). He contended that these Silurian strata pass *conformably* below, and are overlain by, the metamorphic rocks to the east, and inferred that this metamorphic series must belong to the same system. This interpretation meant a radical change in the geological map of Scotland, for the area occupied by these altered strata amounts to about 11,000 square miles.

Professor Nicol, on the other hand maintained that no conformable upward succession from the fossiliferous limestones and associated strata to the overlying schists is to be found. He held that the line of junction is a line of fault "everywhere indicated by proofs of fracture, contortion of the strata, and powerful igneous action."

It fell to the lot of Archibald Geikie to traverse rapidly with Murchison the line of junction in the county of Ross, where, owing to stupendous inversions and overthrusts, the prevalent dip of the fossiliferous strata and the Eastern Schists is towards the east-south-east. He was misled by the apparent superposition and especially by certain deceptive sections in which the Eastern Schists rest with similar dip and strike upon the undisturbed Silurian rocks. Eventually he accepted Murchison's interpretation.

The results of these traverses were embodied in a joint paper communicated to the Geological Society, London, in 1861, 'On the Altered Rocks of the Western Islands of Scotland, and the North-Western and Central Highlands.'

Murchison's interpretation of the structure was therein described and illustrated by sections in such convincing form that it met with general acceptance for many years.

In 1878 the controversy was reopened, and Murchison's position was shown to be untenable by several investigators. Dr. Hicks, Prof. Bonney, and Dr. Callaway made important contributions to the problem.

Prof. Lapworth grasped the true solution of the geological structure of that region. In 1883 he began a series of articles in the 'Geological Magazine' on "The Secret of the Highlands," based on his detailed mapping of the Durness-Eireboll region in 1882, but, owing to severe illness, this series was never completed. He therein demonstrated the inversion of the Silurian strata on the east side of Loch Eireboll and the unconformable junction of the basal quartzite with the old Archæan floor on the east side of the fold at Ant-Sron. From his paper on "The Close of the Highland Controversy" ('Geol. Mag.,' 1885), and from the "Obituary Notice of Charles Lapworth," by Prof. Watts and Sir Jethro Teall ('Proc. Roy. Soc.,' 1921), it is clear that he recognised that the Archæan Gneiss had been driven over the fossiliferous quartzites on Ben Arnaboll by a gently inclined overthrust fault. Along this plane of movement, and at other localities, the original rocks had been crushed and rolled out into types which he termed mylonites. On the shore at Heilim, on the east side of Loch Eireboll, he observed that the serpulite grit had been repeated many times by clean-cut faults, a striking illustration of imbricate structure. All these phenomena were shown in the field in 1883 to Sir Jethro Teall by Prof. Lapworth.

The Durness-Eireboll region was mapped by Peach and Horne in 1883-84, when they reached conclusions practically identical with those of Prof. Lapworth regarding the stratigraphy and metamorphism of the rocks, in complete ignorance of his results (see "Close of the Highland Controversy," p. 98, 'Geol. Mag.,' 1885). It was then proved in the course of the geological survey work that under extreme lateral pressure the rocks behaved like brittle, rigid bodies; they snapped and were driven westwards in successive slices, so that crystalline gneiss and schist are made to rest upon fossiliferous strata of Silurian age. It was further shown that the Eastern Schists were driven westwards by the Moine thrust—the most easterly and most powerful of the series—for a minimum distance of ten miles over all underlying thrust masses, till they rest directly upon the Silurian (Cambrian) limestone in the Durness basin.

The evidence proving these conclusions was carefully inspected in the field by Archibald Geikie, who had never had an opportunity of examining the Eireboll sections. He was completely convinced that Murchison's interpretation of the structure must be abandoned, and he took the earliest opportunity of making a public declaration to this effect. A report giving the results of the work by Peach and Horne, with a preface by Geikie containing a frank

confession that he had been misled and that he had accepted the conclusions of his colleagues, appeared in 'Nature,' November 13th, 1884.

In dealing with the geological structure of the Silurian rocks of the Southern Uplands, he accepted the order of succession adopted by Nicol and Murchison, and was largely influenced in holding this opinion by the doctrine of Colonies. This theory was introduced by Barrande, the distinguished palæontologist, to explain the intercalation of fossils, belonging to higher zones, in lower portions of the Silurian succession in Bohemia. These precursory fossiliferous bands were termed "Colonies." The distribution of the bands of graptolite shale in the Southern Uplands as mapped by the Geological Survey was regarded as an instance of the precursory appearance of the higher graptolite forms in the Moffat region and their disappearance in the Lead Hills district.

But the researches of Prof. Lapworth furnished the key to the solution of the structure of the Chain. He demonstrated a definite faunal sequence in the graptolites which is persistent though the strata may be inverted by folding. He further proved a great variation in the character of the contemporaneous sedimentary deposits; the black shales of the Moffat region, ranging from Arenig to Llandovery time, about three hundred feet in thickness, being represented in the Girvan area by several thousand feet of sedimentary deposits.

Reference must be made to his tenure of the Chair of Geology for eleven years in Edinburgh University. By his lectures, which were given in a clear and attractive form, and especially by his class excursions, he was an inspiring teacher. Edinburgh is an ideal centre for practical training in field geology, but in addition, at the end of each session, he carried out a long excursion, lasting a week or ten days, to examine special problems in the field. Receptive students who took part in these excursions felt the influence of his personality. He thus describes the impression produced on one of his former students by these practical demonstrations.

"These students rambles and the love of geology which they fostered have dwelt ever since those days in Sir William Herdman's memory, and though he has become eminent in another field of natural science, he has assured me that it was the remembrance of his experience in the Geology Class at Edinburgh and its excursions which led him in recent years to found and endow a Professorship of Geology in the University of Liverpool. It is not always that a teacher lives to see the fruition of his labours. Certainly no incident connected with my professional career has given me keener pleasure than this generous liberality of a former student."

Archibald Geikie's eminent services as an exponent of geological science in his class-books and text-books demand special mention. His experience in the field during his early official career enabled him to test the truth of the principles laid down by Hutton and illustrated by Playfair, regarding the evolution of the earth's surface features. The potency of the ordinary agents of denudation in hollowing out valleys was not admitted by some of the leading

geologists of that time. Even Sir Charles Lyell, who was the great exponent of the uniformitarian school, contended towards the close of his career that the principal valleys in almost every great hydrographical basin "have been due to other causes besides the mere excavating power of rivers."

Geikie was an ardent follower of the views of Hutton and Playfair. He was impressed with the remark of Desmarest when pointing out the value of Hutton's contributions to the natural history of the earth and the physical geography of Scotland: "It is to Scotland that Hutton's opponents must go to amend his results and substitute for them a more rational explanation." Geikie was convinced that Scottish topography furnished no grounds for such opposition. He produced in 1865 his volume on 'The Scenery of Scotland viewed in Connection with its Physical Geology,' which gave an excellent popular description of the Huttonian principles of earth-sculpture as illustrated by the country he knew best. He showed with great clearness and artistic style how the ordinary agents of denudation—rain, rivers, the sea, the wind, and moving ice—acting upon different types of rock, had carved out the distinctive varieties of Scottish scenery. As the volume was based on personal acquaintance with the physical features of the country, and a knowledge of its geological structure then ascertained, it immediately arrested attention. Its success was marked; it ran through three editions, the later editions incorporating the more important advances in Scottish geology.

In view of the success of this volume, he then projected a series of educational works on Physical Geography and Geology to meet the public demand. In 1873 he contributed to Macmillan's elementary science series a primer on each of these subjects—a task which cost him excessive labour. His aim in writing the 'Physical Geography Primer' was to stimulate habits of observation of the common phenomena of every-day experience. It ranks as one of his best educational achievements. These primers were followed by class-books on the same subjects, for which there has been a great demand. The great educational value of these publications is beyond doubt. Their success is due to his teaching being permeated with the Huttonian conceptions of natural forces now in operation and to the exquisite literary form in which the lessons are presented.

These primers and class-books were meant to be the precursors of his great 'Text-book of Geology,' the first edition of which appeared in 1882. This volume was intended for the use of students and professional workers in the science, and so well did it meet the requirements of the time that the whole issue was disposed of in a comparatively short period. One of the valuable features of the book was his analysis of the research done in other countries in each of the great divisions of the geological record. It reflected extensive reading and the careful preparation of brief summaries of the results achieved. He made a strenuous effort to keep each successive edition abreast of the research of the time, and, in dealing with his own record, he frankly abandoned

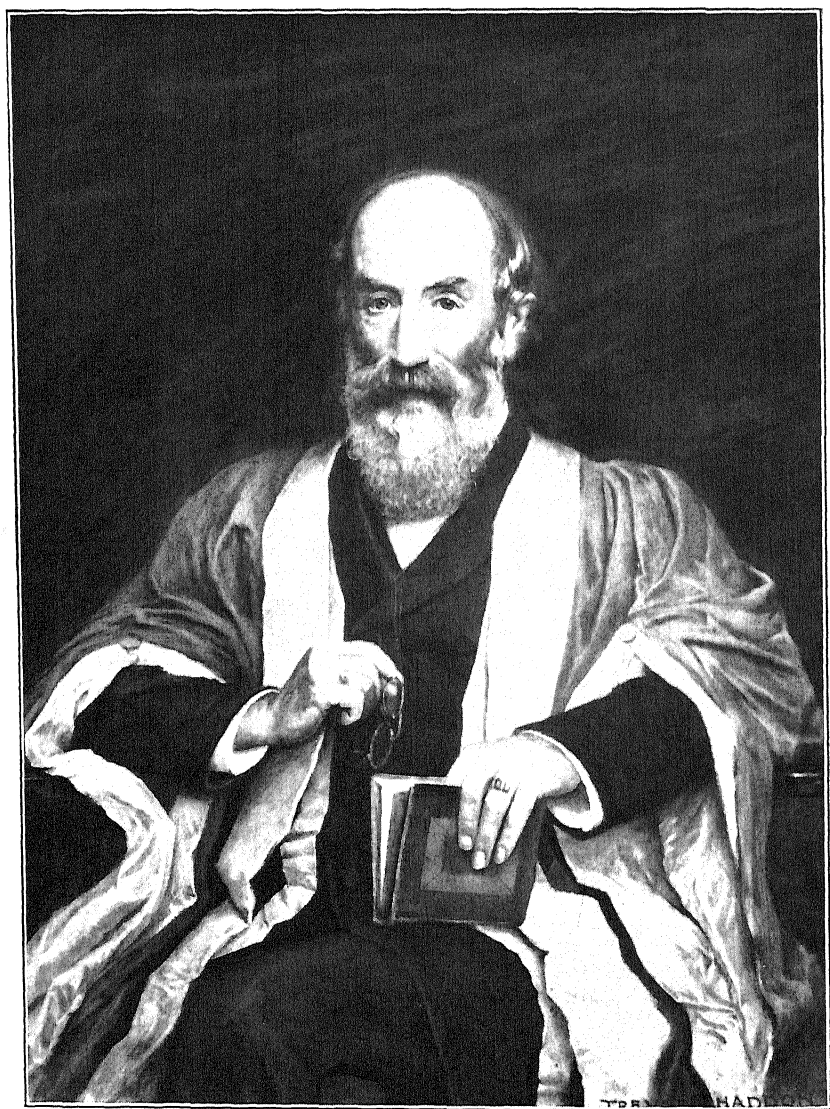
positions which he had previously held when new evidence proved that they were erroneous. The fourth edition, published in 1903, after his retirement from the Survey, is notable for copious references to the geological literature of other countries, which cost him enormous labour. It is also notable for the qualification which he attached to Huttonian teaching, as he thought uniformitarianism had been pushed too far.

“It has often been insisted upon that the Present is the key to the Past; and in a wide sense this assertion is eminently true. . . . While, however, the present condition of things is thus employed, we must obviously be on our guard against the danger of unconsciously assuming that the phase of nature’s operations which we now witness has been the same in all past time. . . . For aught we can tell, the present is an era of quietude and slow change compared with some of the eras that have preceded it.”

A reprint of the fourth edition of the text-book was issued in 1924.

His active brain and fertile pen enabled him to produce several biographies in the midst of other onerous labours. He completed the ‘Life of Edward Forbes,’ begun by Dr. Wilson; he wrote the ‘Memoirs of James David Forbes,’ of Murchison, of Ramsay, who succeeded Murchison as Director-General of the Geological Survey. The Life of Murchison is of permanent interest to geologists from his vivid sketches of the Founders of Geology in Scotland and England, and from his description of the achievements of Sedgwick and Murchison in establishing the Cambrian, Silurian and Devonian systems.

J. H.



T. D. R. HAD.

THOMAS GEORGE BONNEY—1833–1923.

RARELY can there have been a scientific man so many-sided as Thomas George Bonney; mathematician, classic, theologian; by turns college tutor and Alpine traveller, Whitehall preacher, Rede Lecturer, and Professor of Geology, journalist and artist; writer on popular subjects like architecture and landscape and of scientific papers in the most precise style: organiser, teacher, climber, and critic. Industrious and capable, he was not averse from undertaking tasks that most men would have shrunk from; methodical and purposeful, he carried through whatever he undertook. As a result he put to his credit over 200 scientific papers, an average of about four a year for an active period of more than half a century, wrote a shelf-full of books, a long series of scientific articles, and several volumes of sermons; yet he found time to edit or write supplements and appendices to the works of many of his friends.

In addition to all this, as Tutor and Lecturer in a large College, or as Professor in another University, he for many years sent out a stream of pupils who accomplished a vast amount of original geological research and filled many of the more important geological posts in the country.

Of Huguenot family, son and grandson of clergymen, he was born in 1833, the eldest of the ten children of the Rev. Thomas Bonney, headmaster of Rugeley Grammar School and perpetual curate of the small neighbouring parish of Pipe Redware. Educated at first at home, where he began to acquire his taste for natural history, he went to Uppingham at about 14, obtained a school exhibition and proceeded to St. John's College, Cambridge, where he soon gained a scholarship. He graduated as 12th wrangler in 1856 and obtained a second-class in the Classical Tripos in the same year, but was prevented by his health from going on to the Theological Tripos, as he had intended.

After some geological work in the South of England and a long vacation in Switzerland, he was appointed mathematical master at Westminster School, where he took orders. He was elected in 1859 to a Fellowship at St. John's, which he held to the end of his life, and was recalled to Cambridge in 1861 as Junior Dean, thus beginning an academic career which was unbroken for nearly 20 years. He was appointed Tutor in 1868, started a course of geological lectures in the College, and in the following year was appointed College Lecturer on that subject. In this capacity there devolved upon him the main responsibility for geological teaching in the University during Sedgwick's declining years, and Bonney might reasonably have expected to succeed to the Woodwardian chair in 1873. This appointment, however, in those days made direct by the Senate, he lost by a few votes, but he continued his teaching side by side with the new Professor for another seven years. He dealt with a great variety

of subjects, always attracting considerable audiences; but his chief joy was in the teaching, done mostly in his own rooms and with his own instruments, of microscopic petrology, on which he had made himself a recognised authority, thus becoming one of the earliest teachers of that subject in the country.

In 1877 he accepted the appointment of Professor of Geology at University College, London, in succession to his friend John Morris, and for some years conducted the (part-time) work of the chair from Cambridge. In 1881, however, having been appointed Secretary of the British Association, and changes having occurred in the Mastership of his College, he decided to leave Cambridge and settle in Hampstead, where for many years he had the companionship of his sister to keep house for him. The Secretaryship he retained for four years, during which he organised the first meeting of the Association outside Britain—the Montreal Meeting—that turned out in every way a great success, the harbinger of and model for other overseas meetings.

Shortly after his return from Canada he became scientific correspondent to the 'Standard,' and this work making a heavy demand upon his time, he resigned his professorship, which he had held for 24 years, in 1901, having felt for some time that the provision made by the College for teaching material and apparatus was wholly inadequate. He was afterwards made Professor Emeritus. His journalistic work continued till 1905, and then he returned to Cambridge and took the house in Scroope Terrace which he continued to occupy till the end of his life, keeping close touch with his College and especially with the Geological Department in the Sedgwick Museum, where he had a room and saw much of the undergraduate students. After nearly a year of declining health he passed away on December 10, 1923, in his ninety-first year.

He joined the Geological Society in 1860, was Secretary from 1878 till 1884, became President in 1884, and received the Wollaston Medal in 1889. He was also President of the Mineralogical Society, 1884–1886, of the Alpine Club, 1883, of Section C of the British Association in 1886, and of the Association itself at the Sheffield Meeting in 1910. He was elected a Fellow of the Royal Society in 1878, served on the Council on three occasions, and was a Vice-President in 1898–99. Honorary doctorates were conferred upon him by the Universities of Montreal, Dublin, and Sheffield, and he was an honorary or corresponding member of many scientific societies, British and Foreign. He was also an Honorary Canon of Manchester, held the office of Whitehall preacher, and on several occasions delivered the University sermon at Cambridge, once as Hulsean Lecturer.

Bonney's first contribution to science was made in 1862, shortly after his return to Cambridge, when he communicated a paper to the British Association on some flint implements collected by himself at Amiens. This was followed by a research into early French history, from which he concluded that the volcanoes of Central France had been in action in the fifth century.

In 1866 came his first paper on glaciation, a subject in which his interest was lifelong. After his first visit to Switzerland he made detailed studies of the action of glaciers, which he renewed at intervals on his many subsequent visits, and these studies coloured the views which he subsequently developed. He came to the conclusion that both the method and extent of glacial erosion had been misunderstood, that moving ice had not the "plucking" action to which the formation of mountain "cirques" had been freely attributed, and that the power of ice to flow past fragments carried by it, which he was able to demonstrate in favourable localities, was inimical to the execution of extensive erosion. As he put it, ice was "the file rather than the chisel of Nature." For this reason he strenuously opposed the views of Ramsay and others, that tarns and even larger lakes had been excavated by glacial erosion, bringing forward arguments founded on the profile of many such lakes in depth, and the peculiar shape of others, such as Como, in which a projection of softer rock had succeeded in dividing the former ice-stream into two, while much harder rocks must, according to the theory, have been eroded away. And yet the writer can recollect his saying that Anglesey would have been much more interesting if it had not been so severely glaciated.

Neither could he believe that Britain had been reached by an ice-sheet from Scandinavia, relying on the deep channel proved by soundings to run parallel to the Scandinavian coast, to ward off any such stream. Controversy on points like these became acute, and Bonney was not inclined to accept the contention of others that polar ice-sheet action must be more powerful than the valley glacier action which he had studied in Switzerland and Norway, and different in nature from it. He found reasons for believing that the chief drifts, and especially the great Chalky Boulder-clay, were due to the action of marine ice in the form of bergs of the ice-foot, though, of course, he agreed that the smaller drift and moraine phenomena resulted from the action of mountain glaciers. For many years after 1877 he was too much occupied with other subjects to write much on glacial problems, but in 1897 and 1898 he began to return to that subject, roused by the newer glacial work which attributed the disturbance and contortion of rocks *in situ* to the pressure of land ice. In this connexion, in company with his friend Canon Hill, he visited Moen and Rügen, and studied the drifts of the German Baltic coasts, coming to conclusions unfavourable to the views of the extreme glacialists. His attitude towards glacial problems was summed up in the address which he delivered to the British Association from the Presidential chair at Sheffield, an address which roused much controversy leading to a final paper (privately printed) on 'Channels attributed to Outflow Streams from Ice-Dammed Lakes.'

Bonney early took an interest in the geology of Cambridgeshire and adopted the theory that the complicated structure exhibited by the Roslyn Hill Pit,

near Ely, was the result of a series of landslips. The problem of the "Cambridge Greensand," with its occasional strange, far-transported blocks of stone, also attracted him, and he eventually wrote a short guide to the geology of the neighbourhood, which was much used by students until replaced by the Survey Memoir.

Bonney's first interest in petrology is evidenced by a paper in 1876 on the columnar, fissile and spheroidal structures, in which he connected all these together as manifestations of shrinkage during cooling, under varying conditions regulating the escape of heat. But by this time he had vigorously taken up the new microscopic study of rocks. What led to this is not known. He was certainly acquainted with Sorby, pioneer of this branch of research, with Zirkel and the Abbé Renard, and with Allport, the Birmingham naturalist, who for some years had been making his own rock-slices, and publishing his results since 1869. His first study on these lines was of an ultrabasic rock, lherzolite, which presented considerable difficulties in the identification of its minerals, that were successfully overcome. Many other rocks of this type, such as picrite and peridotites like dunite and scyelite, and other rocks with uncommon minerals or unusual combinations of them, like eclogite and euphotide, luxulyanite, trowlesworthite, rocks bearing glaucophane, antigorite and enstatite, and the riebeckite-bearing rock of Mynydd Mawr. He also studied such rock structures as cone-in-cone, nodular and lithophysal structures, and even the "fulgurites," produced by the partial fusion of rocks by lightning on mountain peaks and other exposed situations.

His knowledge of the ultra-basic rocks naturally led to the examination of serpentines, rocks exhibiting little structure even under the microscope, and only the ghosts of minerals, usually hydrated. At this time these rocks were placed among the metamorphic rocks and considered to be associated with and derived from the alteration of limestones. This view he strongly combated, collecting specimens from the Lizard, the Alps and Italy, South Scotland, Anglesey, and many other localities, and making a close study of their occurrence and characters in the field. Finding in basic and ultra-basic rocks that the olivine was in all stages of alteration into the mineral serpentine, he searched his specimens in the light of this observation, and succeeded in showing that similar pseudomorphs were occasionally to be recognised in the serpentine rocks, generally in the higher stages of change. He thus proved that the majority of these rocks were normal, ultra-basic rocks, dunites, lherzolites and other peridotites. Bastite, a serpentinous modification of rhombic pyroxene, was also found in some of these rocks; but, while admitting this change, Bonney was not prepared to admit that lime-pyroxenes or amphibole, still less feldspars, could be similarly changed, owing to the amount of alumina and other ingredients that would require removal. He could not himself find

evidence of such changes, and did not admit the validity of the structural arguments which had led other workers to admit them. He studied the rocks so frequently associated with serpentines, particularly gabbros and hypersthene rocks, and maintained that they were not genetically related, but belonged to distinct epochs of intrusion.

Bonney shared with Allport, Judd, and others in the struggle to attain a rock classification which should be both natural and self-sufficient, and should avoid dependence on theoretical views, some of which might at any moment turn out fallacious. Particularly they combated the German use of age as an essential factor, since microscopic study showed them that many of the differences between pre- and post-Tertiary rocks were due to mineral alteration, resulting from hydration, devitrification, and other time changes.

Mr. (now Canon) Edwin Hill, a Fellow and Tutor of St. John's, had submitted to Bonney specimens collected by himself in Charnwood Forest in Leicestershire, and it was decided that the two should study the area together. The result was three papers in 1877 to 1880 and several others in later years, closing with a last one in 1915. This area of igneous and sedimentary rocks had been mapped by the Geological Survey as of a Cambrian age, but the only direct evidence of age showed no more than that they were pre-Carboniferous. The main facts as to order and relationship were established, and, though the authors were at first inclined to correlate the rocks with the Ordovician volcanic sequence of Lakeland, they ultimately came to regard them as pre-Cambrian. The igneous rocks presented considerable analogies with the "porphyroids" of the Ardennes, and, influenced by the opinion of French and Belgian workers, Bonney and Hill regarded them as pyroclastic in origin. Later, however, as Bonney's experience widened and he grew to rely more on his own observations than on those recorded by others, and as he studied the Ardennes for himself, he very considerably modified these views. The authors showed that, as a matter of fact, the rocks had undergone little real metamorphism, although their structures had been considerably modified by pressure. Unfortunately the papers which contained their results were not very lucidly written, mainly on account of the amount of new matter which had to be described, and it has not been easy to ascertain how extensive was the addition made by them to knowledge of the region. In many cases, indeed, it is only when the ground has been carefully worked over and many facts re-discovered, that it is possible to realise how great was the task accomplished by these pioneers.

While Bonney was investigating Charnwood Forest Hicks had worked his way down through the Ordovician and Cambrian Rocks to the underlying pre-Cambrian at St. Davids—a piece of fascinating research which cast a spell on that of British amateur geologists for a decade. Bonney's growing reputation and his specially valuable experience in Charnwood brought him specimens for report from the chief of the new pre-Cambrian areas as they were opened out,

St. Davids, Carnarvonshire, Shropshire, Anglesey, and later the Scottish Highlands, were tackled in turn, and specimens submitted to Prof. Bonney for his opinion as to their age and origin, sometimes under conditions which did not give the new methods a fair chance. Therefore in few cases was he content to give his opinion without visiting the area for himself, so as to grasp the problems at issue and the interpretation which would be attached to his pronouncements. In some instances, as in Carnarvon and Anglesey, in the Scottish Highlands and the Channel Islands, he found it necessary to make prolonged and detailed studies on the spot, and published papers either by himself or with the co-operation of Mr. Hill or others. This work showed the undoubted existence in these areas of volcanic and plutonic complexes, associated sometimes with unaltered and sometimes with metamorphosed sediments, unconformably covered by Cambrian or younger rocks, and indubitably older than the Cambrian of the areas in question. As a consequence he became sceptical as to the ideas of metamorphism which had run riot for several years and had become expressed in the official maps. Particularly was he unable to agree that sediments had ever suffered metamorphism to so high a degree as to melt them down on a large scale and convert them into igneous products. He urged that in the process of making sediments from igneous materials there had been much selection, sorting, and elimination of material, so that no ordinary type of sediment could be converted into any ordinary type of igneous rock unless the missing substances could be restored to it. The extreme metamorphic view, that there was a "cycle of metamorphism," had been espoused and strongly advocated by Green in his fascinating work on 'Physical Geology,' and Bonney took a great and somewhat malicious delight in destroying one by one the instances used in the chapter headed "General View of the Crystalline Rocks" to support the proposition that sediments could pass by gradual stages into thoroughly crystalline and massive rocks, indistinguishable from primary products of magma consolidation. Much attention was paid by Bonney to the detailed study of contact metamorphism, whenever opportunity arose, and among other cases he collaborated with Allport in an important memoir on the effects produced by the intrusion of the granite of New Galloway.

This work fitted in with the views that he had been gradually formulating during his almost annual visits to the Alps and other Continental regions. Owing to the fact that highly crystalline rocks of foliated character are often in mountain ranges found to rest with apparent conformity on rocks of undoubted Secondary or Tertiary ages, it had been assumed that these were younger, and that rocks of all ages might by metamorphism become converted into highly crystalline schists and gneisses. Bonney, familiar with the highly disturbed character of Alpine structure, clearly saw that this "order of superposition" was untrustworthy, and, in paper after paper, described rocks of this nature, both as to their petrological characters and their field relations, com-

paring them with those of known pre-Cambrian age in Scotland, Anglesey, and elsewhere. On these grounds he concluded that such intensely modified rocks were of great antiquity, and that the evidence adduced as to their transition into normal sediments was not reliable. As an instance, one of his latest papers (with Mr. Winwood) put back the age of the Carrara marble, which had been supposed to be of Triassic date. But the most striking case was that of the schists of the Lepontine Alps, which yield distorted and partially crystallised fossils, used as an example of high metamorphism of Secondary rocks. This Bonney strongly controverted on the general grounds of his own work. Visiting the areas in question and making a detailed study of the rocks in dispute, he was able to prove conclusively that, while the rocks were undoubtedly partially crystalline, their minerals were not those of the ancient schists and gneisses, but were a set characteristic of a low grade of metamorphism, and thus gave no support to the cause they had been supposed to aid. This admirable piece of work had a great influence on the views of his contemporaries and confirmed its author in his own attitude towards the foliated rocks.

Bonney was of opinion that too much influence had been attributed to pressure and the general effects of dynamometamorphism, and, though he frequently described instances of the effects of pressure in directing mechanical and minor chemical changes, he held that this cause had been greatly exaggerated by its adherents, a view to some slight extent modified in his last years; though he never departed from the opinion that intensity of metamorphism was, in the vast number of cases investigated by himself, a function of an early stage in the Earth's geological history.

Though an early and active member of the Alpine Club and a close friend of Whymper, the Mathews, and other distinguished Alpinists, and though occupied with so many of the problems of Alpine geology, Bonney did not take a great interest in the history of the chain as a whole, or in the light thrown by it on mountain building; but he did on one or two occasions give semi-popular lectures or papers on this aspect.

His visit to Canada produced a crop of papers dealing with the more ancient rocks, and one on the circumstances of the occurrence of the so-called *Eozoön Canadense*, in which he showed that, whatever the structure might be, its occurrence at St. Pierre had no relation to the recently described occurrence of it in Tertiary limestone fragments at Monte Somma. Rock specimens collected from all parts of the world were sent to Bonney for study and description. These included rocks from Socotra, the Caucasus, Kilimanjaro, the Andes and Himalayas, the Arctic, and New Zealand. On more than one occasion this led to more detailed study, and particularly was this the case with specimens from the diamond pipes of Kimberley, where he held that the gems were originally contained in eclogite masses, caught up in kimberlite-breccia either from its original home or from a rock containing pebbles of it.

While the major part of his petrological work was concerned with igneous and metamorphic rocks, Bonney did not neglect those of sedimentary origin, as proved by his paper on the Ightham stone, and by his address to Section C at Birmingham, which may be regarded as the pioneer of a class of investigation that has made great strides of late years and bids fair to give new aid in determining the age of rocks and to revolutionise our ideas of the geography of past epochs. In revisiting his old home on the borders of Cannock Chase, he retained his interest in the Triassic rocks, and especially the Bunter Pebble Beds so well displayed there. He studied and collected from these throughout their range, and found that while many of their constituents could be attributed to local sources of origin, the majority and more characteristic types could not be thus explained. These he compared with rocks *in situ* or preserved in the Old Red Conglomerates in Scotland, and claimed a northern origin for them. Distance of transport caused no dismay, for his study of the rounding of pebbles by Alpine streams convinced him that far travel would be required to give to such hard materials their wonderful rounding. Judging by his own experience of Alpine "alluvial cones," and the description by Blanford and others of similar phenomena on a larger scale in Central Asia, he considered that these beds recorded a period of land deposit under arid *régime*. But for the Keuper Marls he was unable to accept an aerial origin and preferred to consider them as having been formed in lakes under semi-desert conditions.

An entirely different line of work was for the Coral Reef Committee of the Royal Society, to which he acted as Chairman, and organised, with aid from Australia, the boring through the Atoll of Funafuti. He edited the Report, which, in spite of the triumph which it afforded to Darwin's theory, was so soberly and restrainedly worded that it was hardly realised how much had been accomplished by Sollas and David in the way of crucial proof.

There is not space to do more than mention numerous books of his own, his scientific appendices to many books of travel, his text-books and special monographs, his volumes of sermons, and his published outline sketches which recall his friendship with Elijah Walton, his studies with him in the Alps, and his descriptions of scenes in the volumes of Walton's published sketches. One of the last pieces of literary work he attempted and brought to a successful issue was his record of the Philosophical Club, a companion volume to Sir Archibald Geikie's book on the History of the Royal Society Club.

The Sedgwick-Murchison controversy had left a legacy at Cambridge of which a considerable share fell to Bonney. He took no little delight in testing the theories of official geologists, and a good deal of his work was dictated by the desire to correct what he suspected to be erroneous interpretations of fact. But his criticisms were by no means confined to official work. His mathematical training made him very exacting in his judgment of scientific theories, and his wide field experience and very extensive acquaint-

tance with the various branches of his subject, coupled with a ready wit and the faculty of quick, and sometimes even sharp or sarcastic, reply, made him a formidable antagonist in discussion, not only in scientific, but in academic and theological matters. Many new theories and their expounders found an opponent in him, not because the theories were novel, nor even because they were becoming fashionable, but because he demanded that they should be exhaustively proved. He had, as he once remarked, often seen "a new theory rise to heaven like a rocket, all star and sparks, only to fall, alas! to earth, all stick and stink." The name "*malleus erraticorum*," which he punningly bestowed on Crosskey, secretary of the "erratic blocks" Committee, might well have been applied to himself. But he felt that it was only unsound work which would suffer in the clash of expert argument, and that the ultimate truth would always survive, especially as the antagonists would be stimulated by it to put forth their strongest and best efforts in support of their views. Naturally, the method offended his weaker antagonists, but the stronger ones sympathised with his attitude, even while they suffered at his hands.

Tenacious of his views as he was in argument, he was by no means unwilling to put the theories of his adversaries to renewed test, and if he could convince himself, out of his own work, of their accuracy, he had no hesitation in ungrudgingly accepting them, as may be seen from such papers as his later ones on the Lizard or Charnwood Forest.

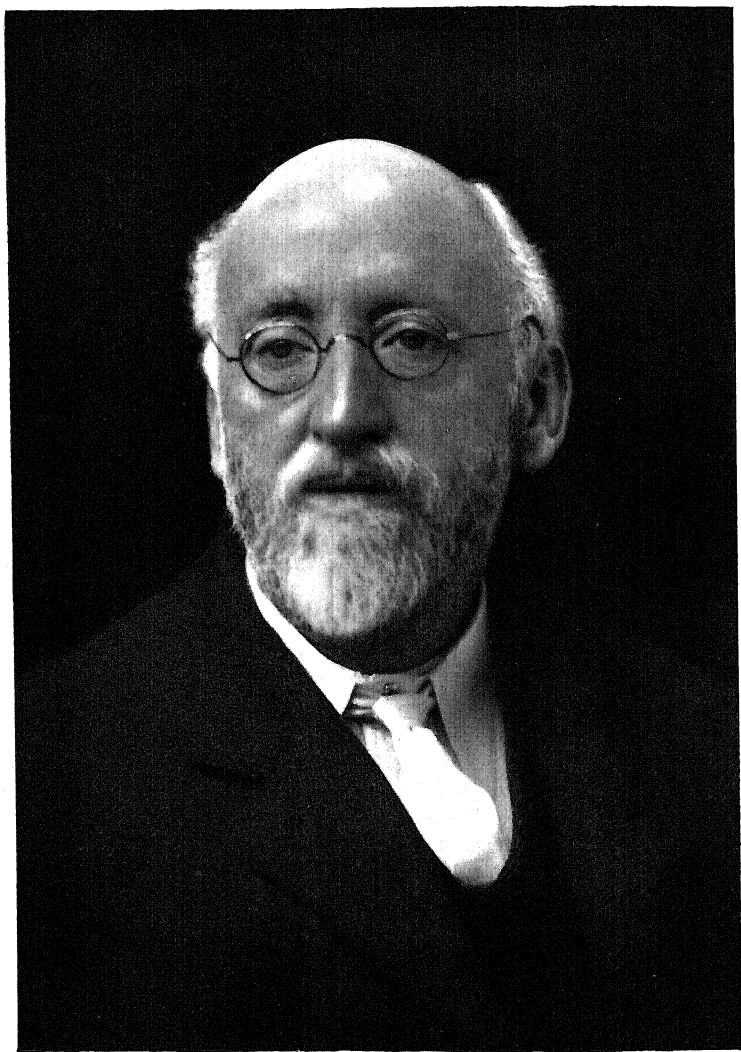
Revealed by his little volume "*Memories of a Long Life*" which he published in his last years, as teacher, college lecturer, and "Don" of a type now passed away, he was especially severe with his pupils. This was partly because he desired to instil into them the necessity for absolute accuracy, but mainly to urge and stimulate them to more and better work. But, inside his academic armour and within the range of his weapons of attack, they found the most tender-hearted kindness, generosity, and good fellowship. His students were always welcome in his rooms, and enjoyed the privilege of his hospitality or of walking with him; and how enjoyable and encouraging were those occasions when the "tutor" was dropped and we might have the pleasure of having him all to ourselves, or the advantage of meeting some eminent friend or foreign scientific man. He was always eager for a student to get out his first paper, for he held that he could then rely upon the *cacoethes scribendi* to do the rest.

His field classes were memorable, whether for short days in the Cambridge neighbourhood, or for longer periods in North or East Wales, or in what he called "West Wales." On these occasions he unbent more than usual and became a mine of anecdote and information, and it was not very difficult to turn him aside from the strict programme for a climb or swim, or some other variant.

Although in most of his papers he played a lone hand, he rejoiced to work in company, and there are an exceptionally large number of joint papers in his list, some worked out with his contemporaries, others with past or present students. He did not share much in larger excursions, such as those of the Geologists' Association, but he was closely connected with two of the more important foreign ones, those to the Ardennes and to Auvergne, and he wrote introductions to them.

As a popular writer he possessed a flowing pen and a charming style, and most of his books and the several publications to which he contributed show a wide and accurate knowledge and are delightful to read. His text-books were not so lightly written, while in his scientific papers his style was severely academic and he generally appeared to have found himself cramped by his endeavour to state his case with absolute fidelity, without exaggerating what was favourable or glozing over what was unfavourable, to pack his sentences as closely as possible with pertinent matter and yet to confine his papers within reasonable limits. As a lecturer, on the other hand, he was perfectly lucid, and his lecture and class work was judiciously selected and carefully arranged so that it was easy to follow, though it kept the note-taker busy. In discussion he was brisk and happy, quick in the uptake, most difficult to corner, and very apt in reply. Many of his retorts are treasured by those who heard them, but a single one must here suffice. On one of the rare occasions in which he coined a new word, it was pointed out that there existed an older term that had been freely used and seemed to fit the case. Like lightning Bonney replied that, though "granitoidite" might have its objections, he still preferred it to the alternative that had been mentioned, one which "conceived in ignorance, was born in shame, and bore the stamp of ignominy on its face."

His field methods were chiefly traverses and sections. He could afford neither time nor patience for detailed mapping, and few of his papers are thus illustrated. Either by faculty or training he seemed to lack the power of "seeing solid" into a map. He further considered that this work could not be undertaken by the amateur, who must take such maps as had been prepared and test them on the ground in the leisure at his disposal. In some of his controversies it might have been well had he adopted this efficient weapon from the armoury of his adversary. Specimens were collected freely and carefully, and as methodically sliced and examined, so that he got together one of the largest collections of rocks and slides ever amassed by an individual, and his knowledge and recollection of what they showed, with his faculty for putting his finger on appropriate specimens in his possession and the information he had about them, were very remarkable. With great generosity he presented rocks and slides to the University of Cambridge. On more than one occasion he excused himself from becoming involved in the mass of literature on a subject, alleging that in many cases the information obtainable was



W. M. Baylis

not worth the search and that it was quicker, easier, and more certain to collect or re-collect the facts for himself.

But when all is said and done, it was in teaching, and all that that implies, that the Professor found his chief occupation and delight, and, even had his original work been but a tithe of what it was, he would have made an unequalled reputation as a maker of tools, whereby he put out his talent at compound interest. His students were never in very large numbers, but with his keen judgment of promise, he was almost invariably able to pick out one or two of exceptional quality, whom he would teach, direct, stimulate and, if necessary, criticise and correct. At one time he was able to point to the then professors at the three greater English Universities, as well as the Director of the Geological Survey and his immediate predecessor, all of them pupils of his own.

It was a great gathering of his students at University College which, shortly before his retirement from the chair there, presented him with his portrait painted by Trevor Haddon. In their hands and through their work, in addition to the extent and brilliance of his own, Bonney's reputation is safe.

W. W. W.

SIR WILLIAM MADDOCK BAYLISS—1866–1924.

WILLIAM MADDOCK BAYLISS, by general consent, was among the most erudite and the most widely disciplined physiologists of his time, certainly in this country, and probably in the world. Within the realm of science his reading was of the widest, his knowledge was comprehensive and his outlook broad. No justice, however, could be done to his worth and his influence in a memoir concerning itself wholly, or even mainly, with his scientific eminence and his intellectual distinction. His quiet generosity, his kindness, his self-effacing modesty and his simple goodness endeared him to all his fellow-physiologists, including among them, as he assuredly would have done, workers not only in the kindred biological sciences but in a wide territory commonly allotted to physics and chemistry.

Bayliss was born at Wolverhampton in 1866, his father being concerned in the manufacture of iron ware. His own inclination was not in the direction of commerce, and he was apprenticed to a doctor; he never finished his qualification, but, on the other hand, he never lost that outlook in life which made him consider the way in which he could best advance the welfare and interests of those about him. He entered University College as a student in

1881, when he came under the influence of Ray Lankester and of Burdon Sanderson, particularly the latter; and so, Burdon Sanderson having been appointed to the Chair of Physiology at Oxford in 1883, Bayliss followed him thither a couple of years later.

At Oxford, Bayliss's undergraduate days were spent at Wadham College, of which college he ultimately became an Honorary Fellow, and in the years which followed the taking of his degree he did some teaching in the Department of Physiology. With that exception his whole active life was spent at University College, London, first under Schafer and then for rather more than the period during which Starling was Jodrell Professor of Physiology. Throughout the whole of that time Bayliss's main occupation was research; in 1912 a special chair of General Physiology was created for him; this he held for the remainder of his life. On several occasions, however, he deputised for Starling; thus during the War it was the more easy for Starling to be absent on active service, and later to be spared for service in a Commission to India.

The earliest researches which Bayliss conducted were on electro-physiology—a fact which was perhaps attributable to Sanderson's influence. Yet at the outset it was electro-physiology of the higher type, that is to say, it was "Physiology" rather than "electrical." The fascination to Bayliss lay, not in the manipulation of delicate instruments, but in the investigation of living processes. Not that Bayliss was uninterested in apparatus—one of the most vivid recollections which any visitor to his home would carry away would be that of his private laboratory, small in dimensions, but packed with apparatus to so great an extent that the guest could but with difficulty make his way around.

Much of Bayliss's work was carried out in collaboration with others, his first contributions to the 'Journal of Physiology' being papers published jointly with Bradford on the electrical phenomena exhibited by the salivary glands. This work, like most work on the salivary glands, was never really driven home; perhaps because there was a good deal of scope for individual interpretation in the results obtained, but I think also for another reason, which is further from the surface, and of which the authors were probably not conscious. In those days, which were about the late eighties, antagonistic nerves held a prominent place in the physiological thought of the time, and the actions of the *chorda tympani* and of the sympathetic respectively on the salivary glands seemed, if fully understood, to form a hopeful avenue of approach to the comprehension of living processes generally. With the march of time, it has become increasingly apparent that the submaxillary gland of the dog, so far from being a type of all living matter, is something very special—almost anomalous—and therefore that the key of life is more likely to be found elsewhere. Just as Langley passed by a natural transition from the histological study of the submaxillary gland to that of its innervation, and

thence to the autonomic system as a whole, so Bradford and Bayliss forsook the salivary glands, the former to carry out researches on the innervation of the viscera, and notably that on the innervation of the pulmonary vessels—a research which has so admirably stood the shock of time. Bayliss, on the other hand, passed to the physiology of the circulatory system—first the electro-physiology of the heart, and then the study of the circulation in the larger sense.

The work on the heart was carried out jointly with Starling, and this was the commencement of a collaboration which extended over most of Bayliss's subsequent life, and was attended with the happiest and most fruitful results. To attempt any analysis of the precise contribution of each collaborator to the result would be an effort certainly invidious, and probably unsuccessful. Like the collaboration of Heycock and Neville on the constitution of alloys, it was one in which the alliance of two men of complementary genius achieved the very highest result; let that suffice, as indeed it did for the Council of the Royal Society, when they sought an *ex cathedra* statement of the researches in the Croonian Lecture of 1904. That lecture was given by "Bayliss and Starling."

The joint work of these authors reached its highest point when they discovered "secretin," for that discovery must, as it seems to me, ever rank as one of the landmarks of physiology—the discovery not merely of a new thing, but of a new process of life. Whilst "secretin" was the high-water mark of their joint work, Bayliss and Starling carried out researches of great importance over a very wide range, *e.g.*, the flow of lymph and the movements of the intestine.

As Bayliss was unencumbered with administration, whilst Starling bore the burden of a large department, it followed that Bayliss had time to spare from their joint work to carry out researches of his own. These at first were concerned with the circulation, and foremost among them may be mentioned his work on vaso-motor reflexes—a subject which embraced a number of researches on more specific subjects, of which some were of first-rate importance, and others (as in the case of all comprehensive work) were of the nature of "spade-work." Included in the work on vaso-motor reflexes, and demanding rather special mention, were his papers on the depressor nerve, on antidromic fibres, on the nature of vaso-dilatation and on the influence of strychnine.

With the advent of the chemical era in physiology, Bayliss became attracted to that side of the subject, and when not engaged in joint work with Starling, he carried out researches on such subjects as ferment action. Before the end of his career, his name became so identified with the chemical outlook on life, that in America, clubs were formed called "Bayliss Clubs," for the object of viewing life from the angle of chemistry.

The great influence which he wielded in this field of thought was due rather to his point of view, and to the completeness of his presentation of it, than to

the importance of his own physico-chemical researches. His active interest in physiological chemistry, so far as it is reflected in his writing, commenced about 1905, by which time the pioneer work in the subject had been largely accomplished. The new century had brought a new spirit into the biological sciences: this spirit found its expression in Bayliss's great book, 'Principles of General Physiology.' Those whom that spirit had already touched found in the book a welcome and concrete expression of much that they felt: to the body of younger men, whose minds after the War were turning to philosophic matters, the book was in the nature of a revelation.

Just as in his earliest researches, his eye had been fixed steadily on life, and not on electrical phenomena, so now, in his later life, it was not the chemistry which gripped him, but the light which it shed on life. Bayliss was not a physiological chemist, he was a chemical physiologist, and it is not without significance that, in the wording of the title ('Principles of General Physiology') of his book there is no direct allusion to chemistry, though it is the most comprehensive work in English on the processes of life, viewed from the physical and chemical standpoint.

So much for the nature of Bayliss's outlook; a further word must be said about its scope. 'Principles of General Physiology' was first published in 1914, and ran through four editions before the date of his death. There can be no doubt of the debt which the physiologists—both teachers and students—of the past decade owe to Bayliss for the work, or of the influence which it has exerted upon the generation which has read it. Rare indeed will it be in the future to find a man possessed of the leisure, the industry, the critical ability, the breadth of view and the experience of research necessary for the production of such a volume. Indeed, just because knowledge is ever increasing, this book may be the last of its kind. It is significant that when the time came to revise the work for the current edition, Bayliss being too ill to undertake the task, each chapter required the skill of an expert in the subject with which it dealt. I imagine there is not one of those experts but would have declared himself incapable of revising the whole work with any degree of authority.

But when all has been said about Bayliss's intellectual outlook, there remains the fact that his usefulness was as much the offspring of his character as of his intellect. In spite of his many distinctions, no man was more approachable, especially by the young. Bayliss loved to have young physiologists about him, and they loved his company. His knowledge, though exhaustive, was never overbearing, and his genius was never frightening—probably because his mind did not work rapidly. He argued a point slowly, sometimes almost tediously, making quite sure of his ground as he went; and in many cases appreciating discrepancies in the argument which might easily be passed over by a less cautious thinker.

Much of Bayliss's work entailed operation on animals, and it was a singular irony that this kindest of men should have been the chosen butt of the anti-vivisection movement. So far did matters go that Bayliss found himself forced to seek the protection of the Court, and the animal, the operation on which was used as the test case, became almost classic.

Bayliss received the honour of knighthood in 1922. He held the following degrees and distinctions: M.A. and D.Sc. (Oxon); Hon. LL.D. (St. Andrews and Aberdeen); Hon. Member of the Danish Academy of Science; Corresponding Member of the Société de Biologie (Paris), and of the Royal Academy of Belgium; Member of the Council of the Royal Society, 1913-15; Croonian Lecturer, 1904; Royal Medal, 1911; Copley Medal, 1919; Baly Medal of the Royal College of Physicians, 1917. He delivered the Oliver Sharpey Lectures in 1918, the Sylvanus Thompson Lectures in 1919, and the Herter Lectures in 1922.

Bayliss's activities during the War were by no means limited to executive and teaching work at University College. He sat on numerous committees of the Royal Society and of the Medical Research Council, but more particularly he was interested in, and worked at, the subject of surgical shock. To meet this condition he suggested the intravenous injection of a 5 per cent. solution of gum-acacia in Ringers Solution. The extent to which the treatment was used may be gauged from the fact that in the summer of 1918 over 5,000 litres was sent forward to the Army through the Base Hospital at Boulogne.

It is perhaps out of place to say much here of Bayliss's domestic life. He was born in 1866, and in 1893 he married Gertrude Starling, the sister of Prof. Starling. Bayliss and his wife lived at Hampstead in a large house—as the houses of professional men go—surrounded by grounds which were a great source of pleasure to him. Their hospitality was unfailing; no physiologist, young or old, but was made welcome—at least if there was such a one I do not know it—and this hospitality extended far beyond the bounds of science. Both were interested in the social conditions of those around them, Bayliss presiding over the local Liberal organisation. Bayliss was a frequent contributor to *Nature* and other periodicals on subjects of passing interest, scientific and otherwise. Such subjects were the use of cocaine, the humane killing of meat, and the like. He and Lady Bayliss were solicitous about the labour conditions of the employes in the family business at Wolverhampton, of which Bayliss was one of the directors, though he did not take a very active part in the company's affairs.

Scientific life was the atmosphere in which Bayliss was thoroughly at home; he loved the occasions on which scientific men met together. He was frequent in attendance at the meetings of the Royal Society, and no meeting of the Physiological Society or Physiological Congress found him absent, until his

last and only serious illness. Of the Physiological Society he was for many years one of the secretaries, and, on the death of Augustus Waller, Bayliss became its treasurer.

In Bayliss's scale of values pageantry stood for little ; science, and especially scientific fellowship, for much. The relative importance of things to him is illustrated by a story, which is in keeping not only with his view of what is important, but with the simplicity of his mind. Summoned to a ceremony of public honour to himself, having a claim of precedence such as few would think of questioning, Bayliss sought to excuse himself on the ground that the Physiological Society met on the date appointed. His innocence of ceremonial convention was as characteristic as his devotion to the Physiological Society, which held, indeed, a central place in his interests.

Some of us have visual memories, and to such visions will arise of Bayliss in situations of many sorts, in the laboratory, at Society meetings, in his home, entertaining his friends, taking part in the discussion of some controversial scientific problem, presiding at a committee where widely different opinions are being expressed ; sometimes mirthful, sometimes serious, sometimes pensive ; but through all moods and in all circumstances there ran one common factor of even-handed equanimity and good humour—there ran the desire for liberality to those less favoured in circumstances than himself, the desire for generosity towards those with whom he differed in scientific discussion, the desire for helpfulness to those who sought his aid. No man more consistently esteemed others more highly than himself.

J. B.



Crundener

ARTHUR DENDY—1865–1925.

ARTHUR DENDY was born on January 20, 1865, at Patricroft, near Manchester. His father was the Rev. John Dendy, B.A., a Unitarian Minister, and his mother was the daughter of another Unitarian minister, the Rev. John Kelly Beard, D.D., so that Arthur Dendy inherited a tradition of culture from both sides of the house. His mother seems to have been an exceedingly clever, well-educated woman, who had enjoyed exceptional educational advantages, owing to the broad and liberal outlook of her father.

When Arthur Dendy was three years old the family moved to Worsley, and the first school which he attended was that of Mr. Clegg at Eccles, near Worsley; subsequently he entered Manchester Grammar School, and then in due time he matriculated into Victoria University (then Owens College). As was to be expected from his early training and associations he was at first on the classical side of the college; but he never evinced much taste for ancient literature. In the course of his college career he came into contact with that brilliant pioneer of modern embryology and morphology, Prof. A. Milnes Marshall, and was fired by him with an enthusiastic devotion to natural history which lasted for the rest of his life. He was the first alumnus of the newly established Victoria University to receive the D.Sc. degree.

After taking his degree he went to London in 1886, when only twenty-one years of age, in order to assist Dr. S. O. Ridley in working out the Challenger Collection of Sponges. When Mr. Ridley retired from the British Museum next year, Dendy was given a permanent position on the staff and had entire responsibility for the Challenger Sponges. Accidents of this kind often determine the whole trend of a scientific career, and sponges were throughout Dendy's life the main object of his researches and evidently had, of all the animal groups, the strongest hold on his affections.

In 1887, he was asked by Spencer, recently appointed Professor of Zoology in Melbourne, to become his demonstrator. He accepted this post and sailed for Australia in 1888, and he remained on the staff of Melbourne University till 1894. His fiancée, Miss Ada Courtauld, followed him to Melbourne, and they were married there in 1888. In 1894 he was appointed lecturer in Biology in Canterbury College of the University of New Zealand, which is situated in the town of Christchurch. This post was subsequently transformed into a professorship, which Dendy held till 1903, when he was appointed Professor of Zoology in the South African College, at Capetown. In 1905 he was appointed to the chair of Zoology in King's College, University of London, and he remained in this post until his death in 1925.

For some years he had suffered from intestinal trouble, and finally in April,

1925, he determined to undergo an operation. This was performed with apparent success, but he succumbed two days afterwards to unforeseen complications which suddenly supervened. His many friends may derive a melancholy consolation from the information that the lesions disclosed by the operation were so serious that had this never been performed his life could not have been prolonged for more than three or four months. He is survived by his widow and three children (one son and two daughters). The elder daughter married Mr. Austen Lane Poole, Fellow of St. John's College, Oxford, and lecturer in History; the younger became the wife of the Rev. E. Leeke of Hemel Hempstead, whilst the son settled in South Africa.

If Dendy had been asked what he considered his most important contribution to knowledge, he would undoubtedly have referred to his investigations on the morphology and classification of sponges. This group was the subject of his first research, and his last paper which is about to be published in the 'Quarterly Journal of Microscopical Science' also deals with sponges.

The sponges, however, form so isolated a group, which throw so little light on the origin and evolution of the higher groups, that his other researches awakened a far wider interest amongst zoologists in general. Amongst specialists in sponges, he enjoyed a high reputation, and will be long remembered by them for two outstanding accomplishments, viz., (1) the thorough investigation and classification of the Calcareous sponges; (2) the study of the development of the spicules of Siliceous sponges, and the consequent re-classification of the Monaxonida. He gave strong reasons for believing that amongst Calcareous sponges the passage from the thimble-shaped "syconid" chamber to the rounded "leuconid" one had been made many times, and that the crust or "cortex" of the sponge with its varying arrangements of spicules afforded a better basis for classification than the shape of the chambers. Amongst Siliceous sponges he showed that "tetraxonid" and "monaxonid" sponges were really variants of the one type, and should be included in one order.

Though primarily interested in sponges, Dendy regarded his sojourn in the Southern Hemisphere as imposing on him the duty of studying the peculiar and interesting forms of life found there. Whilst in Australia he found and described an egg-laying species of *Peripatus*, that most primitive of all arthropods, a far-reaching discovery which has not yet been fully followed up. The late Prof. Adam Sedgwick registered one of the greatest advances in comparative anatomy when he elucidated the structure and development of the Cape species of *Peripatus*, and thus placed the mutual relationships of the Annelida and Arthropoda on a sound basis. But the eggs of this species undergo their complete development within the maternal body, and the earlier stages have become profoundly modified in consequence; and by the study of these, Prof. Sedgwick was led to strange and, we cannot but think, erroneous conclusions. If and when Prof. Dendy's discovery is followed up, we shall be in

a position to correlate much more accurately the development of *Peripatus* with that of other Arthropoda.

Dendy described also the interesting land nemertines and planarians characteristic of Australia, and the ferocity and voracity of the latter group, though apparently defenceless animals, were vividly described in a paper which the writer of this notice heard him deliver to the London Zoological Society. When, however, he migrated to New Zealand, he laid all zoologists under the deepest obligations of gratitude to him for his fine work in collecting, preserving and examining the developmental stages of that primitive reptile the tuatara (*Sphenodon*), one of the relics of the Mesozoic fauna of New Zealand. The development of this form, by far the most primitive type as yet described amongst the Reptilia, was partly studied by Dendy himself and partly by his pupils after he became professor in London. The results of these researches threw valuable light on the primitive segmentation of the brain, the nature and development of the pineal eye, and the homologies of the ear ossicles. In addition to this important subject, Dendy discovered and described an extraordinary type of floating hydroid (*Pelagohydra*) the structure of which enables us to understand the origin of the Siphonophora.

Prof. Dendy was a really splendid lecturer who created much enthusiasm amongst his students and built up a first-class school. He was elected a Fellow of the Royal Society in 1908, and served on the Council during the years 1916-1918. He was appointed a member of the Committee to investigate Grain-pests, a subject of first-class importance during the War, when the depredations of weevils and other insects were causing much anxiety by their effects on our stores of food. He discovered that by enclosing the grain in air-tight bins these insects could be destroyed.

He was a Fellow of the Linnean Society, and was Zoological Secretary of that Society from 1907 to 1912. He took an active part in the affairs of the University of London, and for the last four years of his life he was a member of the Senate.

During his later years he took a greater interest in the wider implications of biological science, and his book 'The Outline of Evolutionary Biology' ran to three editions, and in 1924 he published 'The Biological Foundations of Society.' One of the last places where the writer had the opportunity of friendly intercourse with him was at the summer meeting of the Aristotelian Society at Reading, in June, 1924, when he spoke on the inheritance of mental characters. His death leaves a gap in the ranks of London zoologists which it will be difficult to fill.

E. W. M.

FRANK EVERS BEDDARD—1858–1925.

FRANK EVERS BEDDARD was born at Dudley on June 19, 1858, and died in his house at Hampstead very suddenly, of a heart attack, on July 14, 1925. His father, a prosperous business man, had died young, leaving a widow with two children, Beddard and a sister, in comfortable circumstances. Young Beddard was educated at Harrow and New College, Oxford. His early tastes were for collecting moths and butterflies, and he was not specially distinguished at school. Under Rolleston, at Oxford, he took the old undivided course in Natural Science, but paid most attention to zoology, graduating with second-class honours. He was an early devotee of the high bicycle, and made many collecting holiday trips in this country. He also spent most of his vacations on holiday tours in Germany, France and Spain, acquiring a useful knowledge of foreign languages.

Soon after taking his degree at Oxford, he was appointed to the staff of naturalists engaged, under Sir John Murray in Edinburgh, in working out the collections of the "Challenger" Expedition. Beddard was assigned the Isopod Crustaceans, and the results of his work appeared in vol. 11 (1884), and vol. 17 (1886) of the "Challenger" Reports. They were recognized as accurate and careful work, and at once gave their author an established rank among descriptive zoologists. In Edinburgh, also, he began to take an interest in earthworms, the group in which his most important work was done.

In 1884 he was appointed Prosector to the Zoological Society of London, a post which he retained until he retired on pension in 1915. Soon after coming to London, he married, and the young couple settled first at Ealing and afterwards in a flat in London. Disparity of tastes, however, made the *ménage* unsuccessful, and after some years Beddard, with the son and daughter of the marriage, lived with his mother until her death in extreme old age, after which the three again settled in London. These domestic circumstances are related because they were the first cause of a sense of grievance against life which increased with age, until it became almost a mania of persecution. Apart from the subject of his wrongs, Beddard was a charming companion, with a fund of amusing anecdote that made him a welcome neighbour in clubs or social gatherings. For a large part of his life he was a harder worker than persons with more ordinary habits could guess. He rose very early, and was usually at his table in the Zoo Prosectorium before 8 a.m. On many days he lunched at the Gardens, taking his notebooks into the restaurant, then worked until 4, when he went home, wrote until an early dinner, again worked after dinner, going to bed at 9. When the routine was broken by his going to a club to lunch, he already had a good morning's work behind him and could linger in apparent idleness long after the ten-to-four men had ostentatiously hurried away. In later life, naturally, he was easier with himself.

During the greater part of Beddard's tenure of the Prosectorship, there was the unfortunate tradition that the Prosector must always be ready with a paper for the fortnightly Scientific Meetings of the Society. P. L. Sclater, moreover, his official chief, was interested mainly in systematic zoology, and not being an anatomist himself, was over-ready to believe that even a hurried dissection would settle doubtful points in classification. The double pressure led Beddard to produce far too many short papers, and to draw systematic inferences from dissections of single species or even single organs in species. It cannot be said that his output in any part of vertebrate anatomy had a value at all commensurate with its bulk. A volume on the 'Structure and Classification of Birds,' published in 1895, brought together much useful information; one on 'Whales' (1900) was pleasantly written; his volume on 'Mammals in the Cambridge Natural History' was a sound and useful compilation. Several others need not be mentioned.

Beddard's permanent place in Zoology depends on his great 'Monograph of the Oligochæta' (Oxford University Press, 1895) and on the prolonged researches on which it was based. There were already in existence accounts of the group published by F. Vejdovsky in 1884 and L. Vaillant in 1890, but both of these writers had rather neglected the literature on the subject, and subsequent to their volumes very much new information had been obtained by the study of collections from most parts of the world. Beddard had himself dissected and examined nearly all the important types that were known, and had searched the literature carefully. The general account of the anatomy he gave was much in advance of that of any of his predecessors, and his systematic descriptions were clear and well arranged.

For some time Beddard was editor of the 'Zoological Record.' He was lecturer on Biology at Guy's Hospital Medical School, and gave many popular lectures on zoological subjects at the Gardens and in provincial towns. From 1900 to 1903 he acted as Assistant-Secretary to the Zoological Society, and when P. L. Sclater retired was a candidate for the post of Secretary. Notwithstanding his personal popularity and his long connection with the Society, he did not press his claims in face of a general opinion, which he himself frankly shared, that he was not well adapted to the continuous duties of administration of a large organisation. In 1902 he issued a List of his Contributions to Zoology, in which he was able to cite 239 separate papers and nine books. Subsequently he wrote a number of papers, chiefly on reptilian anatomy and on cestodes.

He was elected to the Fellowship of the Royal Society of Edinburgh in 1883, to the Royal Society in 1892, and he also had the Gold Medal of the Linnean Society conferred on him.

JAMES SYKES GAMBLE—1847–1925.

JAMES SYKES GAMBLE was born on July 2, 1847, at 7, Duke Street, Portland Place, London, and he died a few days after an operation at The College Hospital, Haslemere, on October 16, 1925. He was the second son of Dr. Harpur Gamble, R.N., M.D. Edin., and Isabella Sykes. His early years were spent in London and he was educated at the Royal Naval School, New Cross, under the Hon and Rev. Dr. T. Chambers, starting his school career as early as 1855. His mathematical tastes soon developed and in 1863 he was awarded the Silver Mathematical medal, followed in 1864, by the Gold Medal. This same year he obtained a Demyship in mathematics at Magdalen College, Oxford, and commenced residence in 1865. Here his mathematical ability was rewarded by his being placed in the 1st Class Mathematical Moderations, in May, 1866, and he gained a 1st Class in the Final Schools in December, 1868. In the same year he sat for the Indian Civil Service examination, his place in the list being just below that of the last accepted candidate, and, in February, 1869, he received an appointment in the Indian Forest Department, being placed first of the eight successful candidates.

As soon as Gamble had obtained his Forestry appointment he was sent to the great Forest of Haguenau, in Alsace, where he studied forestry under the Inspecteur des Forêts, M. Clément de Grandprey, whom he found a most energetic and inspiring chief. Here with three other of the selected candidates he spent eight months, and then was passed on, in October 1869, to the École Impériale Forestière, at Nancy, as a member of the 46th Promotion. The course at the Forest School at Nancy was one of two years, but when the war with Germany broke out, in July, 1870, Gamble joined a brother who was mountaineering in Switzerland, where he found greater interest in collecting alpine plants than in mountain climbing. After touring through Northern Italy and across the Adriatic to Trieste and Vienna, and so up the Danube, Gamble was recalled by the India Office, and sent with the others to join the men of the next year who were at St. Andrews University.

When peace between France and Germany was signed, in the spring of 1871, he went back to Nancy to complete his course of training and received his orders for India in October. Before leaving for India in November, 1871, he was ordered to report to Calcutta, and was desired by the India Office to take out Wardian cases of Ipecacuanha plants for the Botanic Gardens there. This necessitated a visit to Kew where, under the guidance of Sir Joseph Hooker, he first became acquainted with the Gardens and Herbarium, of which he was destined later on, when on furlough and after he retired, to see so much.

At Calcutta he received orders for Burma, and was sent to Myanoug, then



J. S. Gamble.

the headquarters of the Tharrawaddy Division. The divisional officer was Major W. Douglas, an old friend, who when on leave a little while before, had spent a month or so at Nancy to see the work of the Forest School.

In August, 1872, Gamble was transferred to Bengal. The Lieutenant-Governor, Sir George Campbell, saw Gamble on his arrival in Calcutta, and instructed him to go to the Darjeeling District and report on the plantations both in the hills and in the Terai. Gamble submitted the reports required and remained in charge of Darjeeling till it was subdivided, and was then transferred to be Assistant Inspector-General of Forests.

When in December, 1872, Dr. Schlich, the new Conservator, arrived, Gamble went on tour with him in the district before being posted to the Darjeeling subdivision, where he commenced a valuation survey, the results of which were published in the Bengal Annual Reports for 1872-73 and 1873-74. While the work was in progress he contracted fever in April, 1873, and had to be sent to Darjeeling, but in June he returned to the Terai and completed the surveys.

In January, 1874, he attended a Conference of Forest Officers at Allahabad, drawing up the Report, and in March was given charge of the Cooch Behar District and was promoted by Sir George Campbell to Second Grade Assistant Conservator of Forests, a notification which, for administrative reasons, the Government of India felt constrained to cancel.

From June, 1874, to August, 1876, while engaged in the ordinary work of the Darjeeling Division, he was able to pay two visits to Independent Sikkim and one to the territory recently ceded by Bhutan, as well as to prepare for publication his first and very useful book, 'Trees, Shrubs and Climbers of Darjeeling and Bengal.'

In 1876 he was summoned to Simla to serve as joint Secretary to the Committee which prepared the first Forest Code for India. Soon after he returned to Bengal he was appointed, in April, 1877, Assistant to the Inspector-General of Forests, and went to Simla to take charge, in which appointment he remained until January, 1879.

During the winter of 1877-78 he was engaged with the preparation of the collection of Timber and Forest Produce for the Paris Exhibition of 1878. and while thus engaged brought together material for his admirable 'Manual of Indian Timbers,' published some years later. His interest in wood structure he owed, as he has himself testified, to the inspiration of the distinguished Prof. Mathieu, while a student at Nancy, where the well-arranged collection of woods in the wood *châlet* in the school garden stimulated him to carry out work on similar lines when he went to India.

Sir D. Brandis, who was always sparing in his praise, wrote the following appreciation of Gamble's work in the Preface to his book :—

"After despatching these collections I considered how to utilize the result of our labours for the benefit of Indian foresters, and I proposed to the Govern-

ment of India that Mr. Gamble should be entrusted with publication on Government account of a Manual of Indian Timbers. The result has been the excellent handbook, which appeared in 1881, describing the structure and properties of the timber of 906 species, with notes regarding the distribution of the trees, their rate of growth and other matters. Since then Mr. Gamble has been indefatigable in collecting specimens of timbers not included in his Manual, examining them on the system established in 1877-8 and revising the descriptions previously made. The second edition of 'Indian Timbers,' published by him on his own account in 1902, contains descriptions of 1,450 species illustrated by excellent photographs, which greatly enhance the value of the work. This second edition is an entirely new book; it is based upon the intimate knowledge of the species constituting the Indian forests, which the author had acquired during his long service on the forests of Burma, Bengal, Madras and North-West India. He has greatly enlarged the notes on the geographical distribution and on the economic uses of the different species and has added notes on their modes of growth, their silvicultural requirements and their treatment in the forest. The suggestion made on p. xix of his Introduction, that keys of the distinguishing characters of timbers, as far as they can be seen with the aid of a good pocket lens, be prepared for the trees found on limited areas, should be borne in mind by the authors of local Forest Floras."

Sir W. T. Thiselton-Dyer, a few years Gamble's senior at Oxford, refers to 'The Manual of Indian Timbers' as a unique and classical piece of work, which secures him a position without a rival in the history of Indian botany.

In January, 1879, Gamble acted as Conservator of Forests, Bengal, in place of Dr. Schlich (on leave), and was then able to make tours of inspection in the forests of Chutia Nagpur, Chittagong, the Duars, and Singbhum. During these journeys he lost no opportunity of making collections of plants, and devoted all his spare time to the careful pressing and labelling and writing up of his collections.

In the autumn of 1882 he visited the Santhal Parganas, and on his return was transferred to the Madras Presidency as Conservator of Forests, Northern Circle, taking up his duties on December 22, 1882. This was a new circle which Gamble had to organize, and with Brandis, who was then in Madras, he visited the Nilgiris, seeing the plantations at Ootacamund, Coonoor, etc., later touring the ceded districts, and travelling through Cuddapah into the Kurnool District.

Throughout 1883 he was busy touring his circle in all directions, writing reports and inspecting the progress of forestry work and generally establishing the forest policy on sound and lasting foundations. The following year he was again in the Nilgiris, and later he carried out an extensive tour of his circle in the company of the Governor.

In 1887 he was in England, on furlough, and spent June in Switzerland with

Sir Henry Collett, where, as usual, he made extensive botanical collections; and in July and August he was travelling in Bavaria, the Tyrol and France, with Sir William Schlich and forest students. He then spent the winter working in the Kew Herbarium, and stayed in England until October, 1888. On his return to India he visited Dehra Dun and Calcutta, where he worked in the Herbarium, and reached Madras again on December 31.

Then followed a busy year of touring in his circle, and, as was the case during all his journeys, he constantly added specimens both to his General Herbarium and to his collection of Indian timbers and of botanical specimens of Indian trees. Then came a marked change in his activities, as he was placed in charge of the Forest School at Dehra Dun, in 1890. Here his main duty was the supervision of the training of the Forest Rangers, and he was, no doubt, able to recall much of the excellent Nancy training, to the great advantage of the students at Dehra Dun. This position he occupied during the last nine years of his service in India until his retirement in 1899. In this year he received the C.I.E., and was also elected a Fellow of the Royal Society.

While he was at the school, Mr. J. F. Duthie, then in charge of the Saharanpur Gardens, and Mr. C. G. Rogers assisted him in making additions to his Herbarium, which by this time had reached a considerable size. He also founded the Forest School Herbarium by presenting a set of the plants he had collected in Madras, Darjeeling, and elsewhere.

Throughout the period of his service in India he pursued his botanical researches with indefatigable zeal, and ample testimony to the value of his labours is afforded by the annotated lists of plants of Darjeeling and Bengal, the Gumsoor tract of the Ganjam District in Madras, the Northern Circars and the ceded districts, publications which have both facilitated the labours of his successors and inspired other forest officers to follow in his footsteps.

It may truly be said that few forest officers have made more valuable contributions to knowledge than did Gamble, and his general outlook is well illustrated in a letter to Kew, written as late as May, 1925. "One thing," he wrote, "may, perhaps, be said with advantage, and that is that, as a forester in India, I was strongly impressed with the necessity of a knowledge of forest plants for the proper study of forest silviculture, and made my collections always with the feeling that the idea, which was held by some, that a forest officer need not be a botanist (I may add zoologist or geologist!) was a great mistake."

Apart from the collections he made and received in India, he also collected specimens at the Cape of Good Hope in 1890, and both before and after his retirement he made extensive collections in Switzerland, Italy, Sardinia, Malta, Gibraltar and South Norway. For his more particular studies Gamble selected families such as the *Lauraceæ* and *Bambuseæ*, which needed careful examination of the flowers, and it was his custom to arrange his dissections for future reference

on gummed pieces of paper in capsules or on the herbarium sheets. It was probably also this liking for dissection which attracted him to study Mosses and Liverworts, for it was his intention to study this group of plants on his retirement. His other botanical interests however, chiefly his work on the Madras Flora, did not permit this to be carried out.

As has been already mentioned Gamble, in addition to his Forestry work, which by itself would have entitled him to a very high place in the roll of scientific honour, was also a systematic botanist of rare insight and critical acumen.

The future of his magnificent Herbarium, which contains some 50,000 specimens, and to which the late Sir George King referred in his address to Section K of the British Association in 1899, as "probably the largest private collection of plants ever owned in India," had been exercising his mind for some little time. In May of last year Gamble munificently offered his Herbarium to Kew, as a gift to the Nation, being anxious that it should not be lost or dispersed. This generous gift was gratefully accepted by Government, and the bulk of the collection had been received at Kew before his sad and unexpected death. The whole collection is now stored in the Kew Herbarium and is available for study, and the specimens will gradually be incorporated in the General Herbarium in accordance with Mr. Gamble's wishes. He also presented to Kew his very valuable and interesting collection of hand specimens of Indian Timbers, which have been placed in No. IV Museum, where they can be studied by Forestry students. In addition, a selection of his botanical books, enriched with his own annotations, has been presented to the Herbarium Library by his widow.

To systematic botany his most important contribution was 'The Bambuseæ of British India,' a monograph involving very careful and critical research. His contributions to the 'Materials for a Flora of the Malayan Peninsula' are also of high value; the first of these was the account of the *Caprifoliaceæ* which was published in 1903, and was followed by that of other orders of *Gamopetalæ*. The date of publication of the last part of Volume IV (February, 1909) almost coincided with the death of Sir George King, with whom he had collaborated, and after that the sole responsibility for the continuation of the Flora fell upon Gamble.

In 1915, when he had completed his Malay work as far as *Salicaceæ*, he was asked to undertake the 'Flora of the Presidency of Madras.' He published the first part at the end of 1915 and succeeding parts in the following years; the seventh, containing the *Euphorbiaceæ*, which was in the press at the time of his death, has now been published. Two further parts had been planned by Gamble, and it is to be hoped that arrangements will be made for this valuable work to be completed.

As a forester, an organiser and administrator, and as a systematic botanist, Gamble was equally a man of pre-eminent distinction. To him we owe a very

great amount of new knowledge, not only about the botany of Indian timber trees and their economic value ; he has also added very greatly to our knowledge of the Flora of India as a whole. It should be mentioned that he was one of the founders of the 'Indian Forester,' and acted for some years as Hon. Editor, frequently contributing articles on Forestry or botanical matters, and so serving to stimulate interest throughout the service.

As a systematic botanist his published works, especially 'The Bambuseæ of British India,' published in 1896 as Vol. 7 of the 'Annals of the Royal Botanic Garden, Calcutta' (xvii + 133 + 17 pp., with 119 pl.); 'The Bamboos of the Philippine Islands' (*Philippine Journal of Science*, Vols. 5 and 8); his contributions to the 'Materials for a Flora of the Malayan Peninsula'; numerous botanical papers in the Kew Bulletin and other scientific journals, and in particular the nearly completed 'Flora of Madras,' and his Herbarium, now the property of the nation, afford ample evidence of his outstanding claim to distinction.

Though Gamble's years of retirement were largely devoted to systematic botany, mainly in connection with the Madras Flora, he continued to take a keen interest in Forestry. From the foundation of the Forestry School at Oxford, in 1906, until its establishment on a permanent basis, he delivered an annual course of lectures on Indian Forestry to the students. He also maintained his interest in the more practical side of Forestry at his home at Highfield, Liss, which he purchased in 1897. Here he planted some 72 acres with experimental plots of species of many exotic trees, the seeds of many of which he had introduced. The trees were carefully tended by Mr. and Mrs. Gamble, and he kept exact and detailed records of all the work that had been done on the plantations. The collection of hardy Bamboos, and two trees of *Quercus semicarpifolia*, raised by Gamble from seed and the only ones in Great Britain, as well as his fine collection of species of Roses, may be mentioned among the plants of special interest in his charming and interesting garden.

Both in India and during his retirement he spent an active life, wholly devoted to service. His conscientious work and his exactitude in classification will stand as a lasting memorial to him in his published works.

A. W. H.

SIDNEY HARRIS COX MARTIN—1860-1924.

SIDNEY MARTIN was the second son of the late John E. Martin, of Jamaica, and was born in that island in April, 1860. He entered University College, London, in 1876, and remained in close association with that institution until the day of his death, when he was Professor of Clinical Medicine at the College.

In his student-days he was an active member of the Students' Medical Society, which then included Victor Horsley, Henry Maudsley, Stanley Boyd, Frederick Mott, C. J. Bond, F. A. Dixey, and Montague Murray.

After taking his degree Martin devoted himself to research. His interests lay mostly in the chemical aspects of physiology, and he became a pioneer in chemical pathology. His first paper, on the papaw fruit and its enzyme papain, appeared in the *Journal of Physiology* for 1885. He was also the first to investigate the nutritional properties of polished rice. This was followed by work upon the poisonous proteins contained in castor-oil beans and jequirity beans; and this in turn led to research on toxins and toxic proteins generally. About this time it was usual to attribute many poisonous effects of bacteria to alkaloids, dubbed ptomaines, and Martin's early work did much to destroy the fallacy, for he found that most of the toxins were really of a protein nature.

At this date bacteriology was little more than an infant science, and it showed signs of over-development on its purely morphological side. Martin was, if not the first, one of the first to recognize clearly that knowledge of the poisonous products of the infective agent was as important as recognition of the causative organism itself, and in his Goulstonian Lectures of 1892 he dealt with the chemical pathology, especially of diphtheria and anthrax; and for his Croonian Lectures, delivered before the College of Physicians in 1898, he took as his subject the chemical products of pathogenic bacteria, with special reference to typhoid fever.

For many years Martin was Assistant Physician to the Brompton Hospital for Consumption, and he carried out a large portion of the experimental work undertaken for the new Royal Commission on Tuberculosis. His results were published in 1895, in Part II of their Report, where he showed that bovine tuberculosis was easily transmissible to other animals. The finding of a later Royal Commission—that which sat from 1902 to 1912—that the disease was communicable to man through tubercular meat or milk, was largely based upon Martin's work.

Martin was an original member of the Executive Committee of the Imperial Cancer Research Fund, and continued a member until his death, representing the College of Physicians. In 1923 he became Chairman of this Committee, and

the clear statement of the work carried out in the laboratories of the Fund, which was published in 1924, was probably his last utterance of importance.

In addition to the scientific papers mentioned above, he was the author of a book on 'Functional and Organic Diseases of the Stomach' (1895), and of a text-book on "General Pathology" (1903). He also contributed articles to Quain's *Dictionary of Medicine* and to Allbutt's *System of Medicine*.

Sidney Martin was for two years on the staff of the Middlesex Hospital, and then followed Victor Horsley as Professor of Pathology at University College; later he became Lecturer in Medicine and finally Professor of Clinical Medicine.

He was twice married, in 1888 to Frances Phillips, by whom he had four children, and in 1910 to Constance Emily Coombes, by whom he had one daughter.

Dr. Martin was elected a Fellow of the Society in 1895 and served on the Council from 1919 to 1921.

W. D. H.

JAMES FAIRLIE GEMMILL—1867-1926.

By the untimely death of Prof. Gemmill, in his 59th year, marine biology loses one of its most distinguished representatives in Scotland. Like many other eminent sons of Scotland, Gemmill was country born and country bred. His father occupied Hillhead farm, near the village of Mauchline in Ayrshire; and his education was begun in the village school. His mother, whose maiden name was Leiper, was the great aunt of another distinguished biologist, Prof. Leiper, of the Tropical School of Medicine, London. James Gemmill, who soon showed himself to be a "laddie o' pairts," was transferred to the Kilmarnock Academy from which at the age of 17 he entered the University of Glasgow, and he remained connected with this university for nearly the whole of his professional life; for it was not until 1919, when he was 51 years old, that he was appointed to the chair of Zoology in Dundee.

Gemmill at first specialized in classical studies, and in these he obtained the highest distinction which Glasgow University had to offer: thus he was awarded the Cowan "Blackstone" gold medals in both Greek and Latin, and in addition the Coulter prize for the best essay on a classical subject, which in his case was "Empedocles: his Position in Literature and Philosophy." But

as he once explained to the writer of this notice, he became tired of classics—they did not seem to him to offer scope for further progress and discovery—and so after taking his M.A. with honours, he turned to the study of medicine. But although in this department he also distinguished himself, taking his M.B. in 1894 and his M.D. with honours in 1900, he never attempted to enter general practice as a doctor. His heart was in anatomical and embryological research, and he became the favourite pupil of Prof. Cleland, who at that time held the chair of human and comparative anatomy in Glasgow. Through the influence of Cleland he was appointed Lecturer in Embryology, and though lecturers in embryology in Scotland generally specialize in human embryology, Gemmill gave a much wider interpretation to the scope of his duties. He became interested in invertebrate embryology, and his lectures gave a survey of the whole field of comparative embryology and he succeeded in inspiring his students with enthusiasm for research. His cousin, Prof. Leiper, received from him his training in methods of research. In 1910 Gemmill was awarded the D.Sc. degree for a thesis on the teratology of fish. Undoubtedly, however, Gemmill's greatest service to science was the part which he took in founding the Marine Biological Station at Millport on the Isle of Cumbrae in the Clyde.

The astounding richness of the Clyde marine fauna had long been known; indeed, the Firth of Clyde with its tributary lochs form a regular marine zoological garden. The late Dr. Anton Dohrn, founder of the Naples zoological station, visited the Clyde before that station was begun, and seriously considered transferring his activities to Millport. A fine amateur naturalist of the old school, Dr. Robertson, of Glasgow, after his retirement from business, had settled down in Cumbrae and devoted himself to systematic work. In co-operation with Sir John Murray, of the Challenger Expedition, Dr. Robertson succeeded in getting the "Ark," a wooden building erected on a barge, brought to Cumbrae as a temporary laboratory. This makeshift "station" was visited by the writer of this notice in 1895 and in 1896, and it was on one of these occasions that he first met Dr. Gemmill. A movement was begun at that time to collect funds for the erection of a permanent station, and for this purpose the "West of Scotland Marine Biological Association" was started. In this association Dr. Gemmill became the leading spirit; through his energetic advocacy a large sum was collected, and after a visit to the leading zoological stations of Europe, Dr. Gemmill drafted the plans for the new building, which was opened in 1900. The writer, who worked there during the summers of 1911 and 1913, can testify from personal experience how admirably adapted to its purpose the station was. Dr. Gemmill became President of the Association, and after retiring from that office continued his connection with the Society as Hon. Vice-President.

The new station was started under great difficulties. Unlike its sister station at Plymouth, it had no support from Government or from rich City companies;

the bulk of its funds were derived from the subscriptions of amateur naturalists, and amateurs formed the majority of the membership of the West of Scotland Marine Biological Association. Under these circumstances it was not unnatural that friction should develop between them and the scientific staff. This came to a head in a quarrel at the annual meeting in 1907 and was followed by the resignation of the Director and of most of the professional biologists on the council. It looked as if the young association was doomed to disunion and failure. It was then that Dr. Gemmill, who had been prevented from attending the meeting by illness, stepped into the breach, and by wise and tactful management succeeded in reconciling the amateur and professional elements and so started the station on a career of research and usefulness. One feature which particularly appealed to the writer when he visited the station was the "teachers' classes." These were held in a large well-lit room in the upper storey, specially constructed for the purpose, and were attended by teachers from elementary schools, who thus had an opportunity of studying marine invertebrates alive. No better methods for spreading a love of biology amongst the people in general could have been devised.

Dr. Gemmill devoted himself especially to the study of the development of starfish, for which Millport offered exceptional facilities. Though the main features of the change from the bilaterally symmetrical larva to the radially symmetrical adult had been worked out in the species *Asterina gibbosa*, this species has a modified creeping larva with a shortened development. The changes undergone by the typical free-swimming Bipinnaria larva in its transformation into *Asterias* had never been elucidated, on account of the difficulties of rearing this larva and of its lengthy development. Gemmill succeeded in overcoming these difficulties and in giving a full and satisfactory account of the life-history of this form—an account which will rank as a zoological classic. He likewise described the development of *Solaster*, the sunstar, which like *Asterina*, has a creeping larva, but one of quite a different type; and he demonstrated the unexpected fact that the many arms of this species are not a primitive feature, persisting from times before the typical pentaradiate symmetry of the Echinoderm had been established, but a secondary modification of that symmetry.

During the War naval restrictions prevented Dr. Gemmill from continuing his work on Echinoderms, and he then turned his attention to the development of Actinozoa, and succeeded in clearing up several puzzling obscurities in the life-history of these animals.

In 1919 Dr. Gemmill was appointed to the chair of zoology in Dundee, and in 1924 he was elected Fellow of the Royal Society. He never married, but he had a niece to whom he was much attached, who looked after his house for him at first in Glasgow and afterwards in Dundee. One evening, returning after attending the University Court at St. Andrews, he experienced the terrible

shock of finding this lady—whom he had left in the morning in her usual health—lying dead on the floor. The grief occasioned by this loss brought on a nervous breakdown; he was granted leave of absence by the University, and his many friends hoped that he was recovering, when he was found drowned in the Tay at Dundee.

E. W. M.

INDEX TO VOL. XCIX. (B)

Address of the President, 30th November, 1925, 107.

Azuma (Y.) and Hill (L.) Effects of Ultra-violet Radiation upon Involuntary Muscle, and the Supposed Physiological Interference of Visible Rays, 221.

Bacillus radiculicola, life-cycle of, and relation to host plant (Thornton and Gangulee), 427.

Balls (W. L.) and Hancock (H. A.) Measurements of the Reversing Spiral in Cotton Hairs, 130.

Batten (L.) See Gregory and Batten.

Bayliss (Sir W. M.), obituary notice of, xxvii.

Beddard (F. E.), obituary notice of, xxxvi.

Best (C. H.) The Effect of Insulin on the Dextrose Consumption of Perfused Skeletal Muscle, 375.

Bonney (T. G.), obituary notice of, xvii.

Brull (L.) and Eichholtz (F.) The Effects of Calcium and Potassium Ions on Urine Secretion as studied in the whole Animal, 57; The Secretion of Inorganic Phosphate by the Kidney.—II. Influence of the Pituitary Gland and of the Wall of the Third Ventricle, 70.

Burnett (W. A.) See Cathcart and Burnett.

Buston (H. W.) See Schryver and Buston.

Campbell (J. A.) Ultra-violet Radiation and Metabolism, 451.

Cathcart (E. P.) and Burnett (W. A.) The Influence of Muscle Work on Metabolism in Varying Conditions of Diet, 405.

Cavia, early development of (Maclaren), 230.

Cell-interior, pH and rH of, before and after fertilisation (Needham), 173; Cell-interior, oxidation—reduction potential of (Needham), 383.

Chlorocruorin pigment (Fox), 199.

Ciliary movement, mechanism of (Gray), 398.

Circulation, effect of light on (Harris), 28.

Cotton hairs, measurements of reversing spiral in (Balls and Hancock), 130.

Crew (F. A. E.) See Greenwood and Crew.

Daly (I. de B.) A Closed-circuit Heart-lung Preparation—Effect of Alterations in the Peripheral Resistance and in the Capacity of the Circulation, 306.

Dendy (A.), obituary notice of, xxxiii.

Diabetic metabolism and exercise (Hetzel and Long), 279.

Eichholtz (F.) See Brull and Eichholtz.

Eichholtz (F.), Robison (R.) and Brull (F.) Hydrolysis of Phosphoric Esters by the Kidney *in vivo*, 91.

Embryonic tissues, studies on differentiation of (Strangeways and Fell), 340.

Erythrocytes, method of measuring the diameters of (Millar), 264.

- Fell (H. B.) See Strangeways and Fell.
- Fischer (E.) The Heat liberated by the Beating Heart, 326
- Fox (H. M.) Chlorocruorin: a Pigment allied to Hæmoglobin, 199.
- Furusawa (K.) A Spirometer Method of Studying continuously the Gaseous Metabolism of Man during and after Exercise, 148; Muscular Exercise, Lactic Acid, etc.—Part XIII. The Gaseous Exchanges of Restricted Muscular Exercise in Man, 155.
- Gamble (J. S.), obituary notice of, xxxviii.
- Gangulee (N.) See Thornton and Gangulee.
- Garner (W. E.) The Mechanism of Muscular Contraction, 40.
- Garrett (F. C.) See Harrison and Garrett.
- Geikie (Sir A.), obituary notice of, i.
- Gemmill (J. F.), obituary notice of, xlv.
- Gonadic structure and plumage characterisation in the fowl (Greenwood and Crew), 232.
- Gray (J.) The Mechanism of Ciliary Movement.—V. The Effect of Ions on the Duration of Beat, 398.
- Greenwood (A. W.) and Crew (F. A. E.) Studies on the Relation of Gonadic Structure to Plumage Characterisation in the Domestic Fowl.—I. Henny-Feathering in an Ovariectomised Hen with Active Testis Grafts, 232.
- Gregory (F. G.) and Batten (L.), a Critical Statistical Study of Experimental Data on the Effect of Minute Electric Currents on the Growth Rate of the Coleoptile of Barley, 122.
- Growth of coleoptile of barley, effect of electric currents on (Gregory and Batten), 122.
- Hæmolysis, inhibition and acceleration of (Ponder), 461.
- Hancock (H. A.) See Balls and Hancock.
- Harris (D. T.) The Effect of Light on the Circulation, 28.
- Harrison (J. W. H.) and Garrett (F. C.) The Induction of Melanism in the Lepidoptera and its Subsequent Inheritance, 241.
- Heart, heat liberated by (Fischer), 326.
- Heart-lung preparation, closed-circuit (Daly), 306.
- Hetzel (K. S.) and Long (C. N. H.) The Metabolism of the Diabetic Individual during and after Muscular Exercise, 279.
- Hill (L.) See Azuma and Hill.
- Hines (H. J. G.), Katz (L. N.) and Long (C. N. H.) Lactic Acid in Mammalian Cardiac Muscle.—Part II. The Rigor Mortis Maximum and the Normal Glycogen Content, 20.
- Innervation of muscle fibres (Katz), 1.
- Insulin, effect on dextrose consumption of muscle (Best), 375.
- Katz (L. N.) On the Supposed Pluri-segmental Innervation of Muscle Fibres, 1.
- Katz (L. N.) and Long (C. N. H.) Lactic Acid in Mammalian Cardiac Muscle.—Part I. The Stimulation Maximum, 8.
- Katz (L. N.), Kerridge (P. T.) and Long (C. N. H.) Lactic Acid in Mammalian Cardiac Muscle.—Part III. Changes in Hydrogen-ion Concentration, 26.
- Kidney, hydrolysis of phosphoric esters by (Eichholtz, Robison and Brull), 91; secretion of inorganic phosphate by (Brull and Eichholtz), 70.
- Lactic Acid in cardiac muscle (Katz and Long), 8; (Hines, Katz and Long), 20; (Katz, Kerridge and Long), 26.

- Long (C. N. H.) Muscular Exercise, Lactic Acid, etc.—Part XIV. The Relation in Man between the Oxygen Intake during Exercise and the Lactic Acid Content of the Muscles, 167. — (See also Jetzel and Long; Katz, etc.; and Hines, etc.)
- Maclaren (N. H. W.) The Early Development of Cavia: Note on Associated Remains of Previous Placentation, 230.
- Martin (S.), obituary notice of, xlv.
- Meiotic phase in mammals (Walker), 366.
- Melanism in Lepidoptera, and inheritance (Harrison and Garrett), 241.
- Metabolism and ultra-violet radiation (Campbell), 451.
- Metabolism (gaseous) and exercise (Furusawa), 148, 155.
- Metabolism, influence of muscle work on (Cathcart and Burnett), 405.
- Metabolism of diabetic individual (Hetzel and Long), 279.
- Millar (W. G.) The Diffraction Method of Measuring the Diameters of Erythrocytes, 264.
- Muscular contraction, mechanism of (Garner), 40.
- Muscular exercise, lactic acid, etc (Furusawa), 155; (Long), 167.
- Needham (J. and D. M.) The Hydrogen-Ion Concentration and Oxidation-Reduction Potential of the Cell-Interior, before and after Fertilisation and Cleavage: a Micro-Injection Study on Marine Eggs, 173; Further Micro-Injection Studies on the Oxidation-Reduction Potential of the Cell-Interior, 383.
- Obituary notices of Fellows deceased:—
- | | |
|----------------------------|-------------------------|
| Bayliss, Sir W. M., xxvii. | Gamble, J. S., xxxviii. |
| Beddard, F. E., xxxvi. | Geikie, Sir A., i. |
| Bonney, T. G., xvii. | Gemmell, J. F., xlv. |
| Dendy, A., xxxiii. | Martin, S., xlv. |
- Pituitrin, secretion in mammals (Verney), 487.
- Ponder (E.) A Study of certain Forms of Inhibition and Acceleration of Hæmolysis, 461.
- Proteins, undescribed products of hydrolysis of (Schryver and Buston), 476.
- Robison (R.) See Eichholtz, Robison and Brull.
- Schryver (S. B.) and Buston (H. W.) The Isolation of some hitherto Undescribed Products of Hydrolysis of Proteins.—Part II., 476.
- Sherrington (Sir C. S.) Presidential Address at the Anniversary Meeting, 30th November, 1925, 107.
- Strangeways (T. S. P.) and Fell (H. B.) Experimental Studies on the Differentiation of Embryonic Tissues growing *in vivo* and *in vitro*.—I. The Development of the Undifferentiated Limb-Bud, 340.
- Thornton (H. G.) and Gangulee (N.) The Life-cycle of the Nodule Organism, *Bacillus radicicola* (Beij.), in Soil and its Relation to the Infection of the Host Plant, 427.
- Ultra-violet radiation and metabolism (Campbell), 451.
- Ultra-violet radiation effects on involuntary muscle (Azuma and Hill), 221.
- Urine secretion, effects of calcium and potassium ions (Brull and Eichholtz), 57.
- Verney (E. B.) The Secretion of Pituitrin in Mammals, as shown by Perfusion of the Isolated Kidney of the Dog, 487.
- Walker (C. E.) The Meiotic Phase in certain Mammals, 366.

I. A. R. I. 75.

IMPERIAL AGRICULTURAL RESEARCH
INSTITUTE LIBRARY
NEW DELHI.

Date of issue.	Date of issue.	Date of issue.
23.6.45		
3.9.52		
12.11.59		